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THE VIRUS OF HERPES: ITS IMMUNE REACTIONS AND ITS RELATION TO THAT OF ENCEPHALITIS LETHARGICA.

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THE investigations, of which the following is an account, were undertaken as a continuation of those carried out by the author in 1921–22 (Parsons, McNalty and Perdrau, 1922). The principal reasons for which a virus, which gives rise to somewhat insignificant lesions in man, was chosen for special study, were that it has been alleged to be identical with that of encephalitis lethargica, that it belongs to the class of the so-called filter-passing viruses, and that we have in the rabbit a highly susceptible animal, whose reactions to the virus, both histologically and clinically, are very characteristic.

RELATION TO SPONTANEOUS ENCEPHALITIS OF RABBITS.

It is necessary at the outset to make some reference to the spontaneous encephalitis of rabbits, which has been described within the last few years, and is regarded by some (McCartney, 1924) as a serious source of error in experiments of the nature of those referred to here. Although extensive epizootics of the disease have been described in America, very few cases have so far been recorded in England. In the course of this research the brains of over 250 rabbits, either under experiment or from stock, have been examined histologically by the author or by Dr. Da Fano, and no case of the disease has so far been found, except in the brains of some of those inoculated with a passage virus of encephalitis lethargica supplied by Prof. Kling to Dr. Da Fano. The same negative results have attended a review of old material and an examination of the brains and kidneys of six suspected animals.

A critical review of the literature of this subject has recently appeared in English (Da Fano, 1924), and no attempt will be made to cover the same ground again.

Briefly stated, the main differences between the spontaneous encephalitis of rabbits and the experimental herpetic encephalitis of the same animal are that the former is characterized by a very variable incubation period ranging from a few days to several months, some emaciation, and the presence of peculiar inflammatory foci and of certain parasites in the brain and kidneys of infected animals. The latter is characterized by an incubation period of from 4 to 7 days, the occurrence of a characteristic clinical syndrome (fits, grinding of teeth, salivation, so-called "circus movements," etc.), and the histological appearances of an encephalitis, in which the special features of the spontaneous disease are absent. It may be stated with confidence that the spontaneous disease is not a cause of error in the investigations considered here.

PART I.—INFECTION AND COURSE OF THE DISEASE.

As the strain studied in 1921 (strain H 1) was found to be dead when the present investigation was started, a new strain (H 4) was obtained from some 24 hours old vesicles, which developed on the lips of a middle-aged woman who was suffering from an ordinary cold in the head. Unless otherwise stated this strain has been used throughout this research.

All the strains studied so far (seven in all) have been found to be identical in every way, with the single exception that whereas labyrinthine symptoms were the rule with strain H 1, these symptoms have only on rare occasions been associated with the other strains. Contrary to expectation, the only strain (H 6) derived from a case of herpes of the lips and cheeks, which has recurred at fairly regular intervals in the same area of skin for the last twelve years in a subject 27 years old, has behaved like the majority of the strains studied, six of which were derived from sporadic cases of the disease. It appears, therefore, that from the experimental point of view the sporadic and the recurrent types of herpes cannot be distinguished from each other even by cross-immunity experiments, but that amongst the sporadic ones an occasional strain may be met with, which is characterized by the production of labyrinthine symptoms in the rabbit.

Several of the conclusions arrived at on a former occasion were confirmed by a fresh series of experiments, only one of which deserves special mention. In a single experiment the virus had been found to be killed by subjection to a temperature of 50° C. for half an hour. This is not invariably the case, as the virus has resisted this temperature at least once.

Of the possible methods of infection, only four (*i. e.* cerebral, corneal, nasal and cutaneous) were made use of with a view to studying the dermotropism and neurotropism of the virus, as well as its immunity reactions.

A brief account of certain preliminary experiments is necessary before considering the peculiar immune state which is met with in infections with the virus of herpes, because the several methods of infection and the technique employed appear to determine whether the result will be an acute infection with fatal outcome or a local infection with subsequent immunity.

Cerebral route.—An intracerebral inoculation into a new rabbit of the passage virus (*i. e.* virulent brain kept for not more than a fortnight in 50 per cent. glycerin in saline, pH 7·4 and ground up in saline) has proved

fatal in 39 cases out of 39 *inoculated in series*, but when the contents of the vesicles of human cases of herpes were so inoculated a positive result was obtained 6 times out of 7, the only exception being one out of two rabbits inoculated with the contents of the same syringe which failed to develop any symptoms, but later succumbed to an injection of the passage virus. In the case of fresh herpetic vesicular fluid the first passage usually takes 10 days, the second 7 days, and afterwards 5 days on an average.

Recovery after the onset of symptoms pointing to an encephalitis which was the result of an intracerebral inoculation was never observed. But recovery took place on four occasions when the portal of entry was other than the brain. One of those four animals subsequently relapsed and died of a typical encephalitis.

If rapid intracerebral passages are carried out in series without allowing the virus to remain more than a few days in glycerin, an alternation is noticed between the low figures of 3 or 4 days and the higher ones of 6 or 7 days for the length of time which the virus takes to kill the rabbit. This alternation was found to vary inversely with the intensity and the extent of the inflammatory lesions present in the brain from which the inoculum was prepared. For instance, the brain of a rabbit, which lives for 6 or 7 days after an intracerebral incubation, shows a severe inflammatory reaction over practically the whole brain, and an immediate injection of an emulsion of this brain into a new rabbit proves rapidly fatal (3 to 4 days) and *vice versâ*. But it should be pointed out that the incubation period is reduced by two days if a second inoculation is given 48 hours after the original one. The first inoculation need not necessarily be an intracranial one, as a cutaneous inoculation produces similar results. When an animal dies as the result of an inoculation into the brain, the virus can be recovered not only from the brain itself, but also from the nasal mucosa and from the saliva (7 cases out of 8), though usually in an attenuated form. This fact is demonstrated if the undiluted and unfiltered mucus or saliva is tested for the presence of the virus by application to the scarified cornea. The nasal mucus was obtained by means of an ordinary throat swab, and was immediately rubbed on the scarified cornea of a new rabbit. A sample of the saliva was taken in a similar manner from inside the cheek and was tested by the same method. In 6 out of 8 cases an obvious keratitis, without any conjunctivitis, followed by complete recovery, was obtained. In one case, where nasal mucus was used, a severe keratitis involving the whole cornea developed and was accompanied by an equally severe conjunctivitis. This ultimately led to the production of a fatal encephalitis. Subsequently passages from brain to brain were easily effected.

Levaditi (1922, p. 196) had long suspected the presence of the virus in the nasopharynx of animals suffering from an encephalitis as the result of an intracerebral inoculation, but he failed to demonstrate the presence of the virus in that situation. His failure may possibly be attributed to the fact that he diluted and filtered these secretions. Negative results were also obtained in the course of this inquiry, whenever these secretions were filtered through a Pasteur-Chamberland L 2 candle.

It is worth noting that the cerebrospinal fluid and the anterior chamber

fluid of animals which die as the result of an intracerebral inoculation of the virus are not virulent at all, and do not neutralize the virus *in vitro*.

Corneal route.—The results of corneal inoculations showed interesting differences according to the virus used. The passage virus was inoculated on the cornea of a new rabbit 10 times by rubbing virulent brain on a scarified area. A simple keratitis was obtained twice. In one case only did a severe kerato-conjunctivitis, followed by an encephalitis, result. Completely negative results were obtained 7 times. The type of anæsthetic used in these experiments did not appear to influence the results. On the other hand, the contents of fresh herpetic vesicles from man were inoculated 4 times on the cornea, with the result that a severe kerato-conjunctivitis developed in every case. A spread of the infection to the brain ensued in three of these cases.

Two possible explanations may be offered for the different results obtained with the two viruses:

(1) The infective agent may be in close association with the fragments of brain-tissue, which were rubbed into the cornea, in the case of the passage virus; and in the case of the vesicular fluid, where there is but little cell detritus, the infective agent is dispersed in the fluid. The chances of infecting the corneal cells will clearly be greater if the infective agent be free and not associated with other inert particles.

(2) The virus may have different properties according to its origin. Obtained from the brain it may possess neurotropic properties, and, when recovered from the skin, dermotropic ones. A dermotropic strain obtained from a skin-lesion is more likely to infect a tissue which is genetically closely related, such as the cornea, than is a neurotropic strain like the passage virus.

In favour of the first explanation it may be recalled that in the case of brain emulsion (passage virus), however finely ground up, filtrates through L 2 candles are non-infective, although Levaditi and Harvier (1920[1]) have recorded a successful filtration of their encephalitic virus through a Pasteur-Chamberland L 3 candle. Successful filtration of vesicular fluid in high dilution through L 2 candles has been carried out twice in the course of this inquiry.

There is, however, a considerable body of evidence in favour of the second explanation. Many facts in support of it will be found in the collected papers of Levaditi (1922) and his associates. Teague and Goodpasture (1923) studied two strains of the virus of herpes, and found that a secondary encephalitis after a keratitis was never observed with one strain, but was the rule with the other.

The following experiments lend additional support to this view. The passage virus was transferred on to the skin of some animals previous to the corneal inoculations, and in 4 cases out of 4 inoculated a severe kerato-conjunctivitis resulted. In another set of 4 experiments the tears obtained from 4 cases of severe kerato-conjunctivitis during its acute stage (3–5 days old) were inoculated on the cornea of new animals, and in all 4 cases a severe kerato-conjunctivitis resulted.

It was noticed in the course of these experiments that a simple keratitis without a concomitant conjunctivitis cannot be passed on in series, and that

out of 16 such cases not one developed an encephalitis. Tears from cases of simple keratitis have not been found to be infective. On the other hand, out of 8 cases in which a severe general conjunctivitis accompanied the keratitis, 6 died of a spread of the infection to the brain, and the tears of these cases were highly infective for the first few days. They ceased to be infective, however, as soon as the conjunctivitis began to clear up. The latter usually disappears several days before the keratitis.

Attempts were made at producing a conjunctivitis without a keratitis, but they proved unsuccessful, as a keratitis rapidly followed on the conjunctivitis. The latter was easily obtained by rubbing the conjunctiva with infective tears and with the scrapings of a skin lesion in two cases out of four. When the passage virus was used no lesion resulted in each of two attempts.

The path followed by the virus on its way from the cornea to the brain has been traced in part by various workers. Levaditi (1922, p. 188) and his collaborators have shown its presence in the retina and the optic nerve, when the keratitis is not more than three to five days old, and Teague and Goodpasture (1923) have traced it along the ophthalmic division of the fifth nerve as far as the pons. Some experiments were carried out with the anterior chamber fluid, when the keratitis and conjunctivitis were still acute. Five such fluids were inoculated intracerebrally, and were found to be incapable of producing an encephalitis. When the animals were tested later by the same route with the passage virus one was found to have been immunized. That the immunity was not passively produced is shown by the fact that none of these fluids neutralized the virus *in vitro*, whilst when injected by the corneal route, the same fluid which produced cerebral immunity gave rise to a keratitis. The virus must therefore have been present in an attenuated form in the fluid injected. The percentage of animals which became immunized as the result of a corneal inoculation was only 16·6 (*i.e.* 4 in 24).

Nasal route.—Intranasal inoculations of the passage virus were tried on ten different occasions by rubbing the virus on the nasal mucous membrane with an ordinary throat swab sufficiently firmly to scarify the membrane. Seven animals developed a typical encephalitis and died in from 6 to 12 days; the other 3 were found to have been immunized by the inoculation as they resisted further doses of the virus, given both intranasally and intracerebrally, for periods of 1, 2 and 3 months respectively. The number of animals immunized by a nasal inoculation was therefore 30 per cent. of the total.

Cutaneous route.—Two different methods were adopted for skin inoculations:

(1) The virus was injected intradermally.
(2) An area of skin was shaved and lightly scarified with a blunt knife and the virus was rubbed firmly over that area.

A local reaction in the shape of an inflamed and tumefied area appeared within 24 hours, whatever the virus used (*i. e.* passage virus or fresh human vesicular fluid). Small vesicles were noticed only three times in 42 inoculations, and in all 3 cases passage virus had been used. The local reaction was sometimes very slight, and occasionally disappeared on the second day in the case of the intradermal inoculations. Usually, and particularly in the

case of scarification of the skin, this reaction lasted for several days, and was much more severe with the dermotropic than with the neurotropic strain. The virus is easily recovered from these lesions as long as the inflammation lasts.

Out of 13 animals which received the passage virus into a scarified area of the skin, 10 became immunized against a subsequent intracerebral inoculation. Two developed a typical encephalitis and died in 15 and 17 days respectively. One showed no symptoms and failed to develop any immunity. On the other hand, out of 7 animals which received intradermal inoculations of the passage virus, 6 were immunized, and 1 showed no symptoms and did not become immune. The irregularity of the results of an inoculation of the passage virus into scarified skin led to some further experiments. It was found that on the *depth* of the scarification depended the result to be obtained. For instance, 3 rabbits were carefully and very lightly scarified on the skin of the abdomen, and 5 others more deeply, and the passage virus was rubbed into these areas. Of the 3 lightly scarified rabbits 2 were immunized, and 1 showed no symptoms and no immunity. Of the 5 deeply scarified, 4 developed a typical encephalitis, and 1 became immunized. In all these experiments virulent brain was used which had not been kept longer than a fortnight in glycerine. The total length of the scarifications in the animals lightly scarified was several times greater than that in those more deeply scarified.

Inoculations of fresh human vesicular fluid by deep scarification of the skin were carried out on three occasions, and although the local reaction was far more severe than when the passage virus was used, no immunity and no spread of the infection to the central nervous system was observed in two animals, whilst the third became immunized. Similarly the scrapings of the skin lesions produced in two rabbits by the passage virus were rubbed on the scarified skin of two normal animals. A well-marked local reaction resulted in both, and they ultimately developed a state of partial immunity. Again, a virus, which had been subjected to only one cerebral passage, produced a good local reaction and a state of immunity lasting a fortnight in one and three months in the other of two animals inoculated by scarification of the skin.

The following conclusions may therefore be drawn: (1) That cerebral immunity is best attained by the inoculation into the skin of a neurotropic and not a dermotropic strain. (2) That of the two routes employed, the intradermal one is the safer, although two cutaneous inoculations at intervals of less than 11 days may give rise to an encephalitis, in the same way that two intracerebral inoculations, or a cutaneous one and a cerebral one, at 48 hours' interval reduce the incubation period appreciably by increasing the virulence of the virus. This may be the result of the production of an aggressin in the brain as pointed out further on. (3) That a dermotropic strain (*e.g.* fresh human vesicular fluid) acquires neurotropic and full immunizing powers by a single cerebral passage, whilst a neurotropic strain loses its neurotropism, and therefore its immunizing powers, only in part by a single skin passage. The cutaneous route therefore yielded the highest percentage of immune animals: 77 per cent. for those inoculated by scarification of the skin, and 86 per cent. by intradermal inoculations. These figures refer to the neurotropic strain only as the antigen.

PART II.—IMMUNITY.

The close relation of the dermotropism or neurotropism of the virus of herpes to the question of its immunity reactions is only one of the many peculiarities which differentiate the latter from those commonly found in bacterial immunity.

The term "immunity" is here used to describe the resistance of an animal to an intracerebral inoculation of the passage virus. This virus has been shown to give rise to a fatal encephalitis in 100 per cent. of new animals. A similar inoculation into an immunized animal remains without effect.

The immunity of another tissue has also been studied, namely, the skin, where the passage virus has been shown to produce a definite and obvious reaction in the form of an inflamed area extending beyond the area actually inoculated and lasting usually for several days. But although some fifty observations were made, no general conclusions could be drawn from them, as it was soon found that cerebral immunity does not connote epithelial immunity.

Corneal immunity has not been studied owing to the fact that the passage virus produced a keratitis only three times out of eight in new animals, and although fresh human vesicular fluid always produces a keratitis in new animals, a supply of it is not always at hand, and it was comparatively late in the course of this research that it was found that any dermotropic strain obtained from the rabbit, such as the tears from a kerato-conjunctivitis or the scrapings of a skin lesion, acted on the cornea in exactly the same way as their human counterpart, namely, the contents of herpetic vesicles.

As already pointed out, a state of active immunity can be produced by inoculations of the neurotropic virus into the skin, and also, though more rarely, into the nasal mucous membrane and the cornea. The same result has been obtained on two occasions by an intracerebral inoculation of an attenuated form of the virus, viz. anterior chamber fluid from a case of kerato-conjunctivitis and virulent brain, which had been kept 135 days in 50 per cent. glycerin in saline, to which a piece of chalk had been added. It was in this last case that the longest period of immunity was observed, as the rabbit resisted an intracerebral inoculation given on the 380th day, but succumbed to one given on the 426th day. The period of active immunity following single inoculations, either into the skin or the mucous membrane of the nose, varied between one and four months, the average being three months.

Cerebral immunity as the result of a corneal inoculation was observed four times only and was quite short-lived—under one month in each case.

Of the various methods available to produce a state of active immunity the intracutaneous one is apparently the safest, because so far no case of general infection has been observed from its use, providing the inoculations are given at one sitting, or are repeated at intervals of over eleven days. Attempts were made to attenuate the virus for immunizing purposes by heating at 60° C., but the results were not encouraging.

Numerous experiments were carried out with a view to demonstrating the presence of antibodies in the fluids of the body and in extracts or emulsions of certain organs of immunized animals, either by attempts at conferring a state

of passive immunity in new animals or at neutralizing the virus *in vitro*. All these attempts failed, but some of the results obtained were quite interesting owing to their unexpectedness.

On a former occasion (Parsons, McNalty and Perdrau, 1922) a rabbit was found capable of resisting an intracerebral inoculation of the virus 11 days after the immunizing dose. The earliest appearance of a state of active immunity after a cutaneous inoculation was therefore determined as follows: Two batches of six rabbits each were immunized by cutaneous and intracutaneous inoculations respectively of the passage virus. One from each batch received an intracerebral inoculation of the virus at the same time, and 2, 4, 6, 8 and 10 days later. Those tested at the same time and 4 days later died in 6 days, like the control animals. Those tested 2 days later behaved like animals receiving two intracerebral inoculations at 48 hours' interval and died in 3 days with typical symptoms, whilst those tested on the 6th, 8th and 10th days lived from 3 to 8 days longer than the control animal, thus showing early signs of immunity. On the other hand six rabbits were tested on the 11th day, and all survived an intracerebral dose of the virus.

Experiments for demonstrating the possible presence of antibodies in the blood-serum of immunized animals.—Four rabbits, R 127, 128, 130 and 131, were immunized by intradermal inoculations of the passage virus in the case of the first two and by scarification of the skin in the others. Their immunity was proved by their resistance to subsequent intracerebral inoculations of the virus. A few c.c. of blood were removed from the marginal vein of the ear on the 11th day after the original skin inoculations. The blood was allowed to clot, and the clear serum, without heating, was added to an emulsion of the passage virus. The mixture was kept for 5 hours in the incubator and then injected intracerebrally into new rabbits as follows:

Material inoculated.	No. of rabbit.	Result.		
Serum of R 128 virus	135	Death in 7 days	Typical	encephalitis
,, ,, ,,	136	,, 5 ,,	,,	,,
,, 127 ,,	137	,, 7 ,,	,,	,,
,, ,, ,,	138	,, 3 ,,	,,	,,
,, 130 ,,	139	,, 11 ,,	,,	,,
,, 131 ,,	141	,, 7 ,,	,,	,,
Virus only (control)	134	,, 7 ,,	,,	,,

It will be noticed that in one case (blood-serum of R 130) the fatal result was delayed up to the 11th day. The blood-serum of that rabbit was again tested on the 21st day, with the result that the two rabbits so inoculated (R 143 and 144) died in 9 and 10 days respectively from a typical herpetic encephalitis. The slightly longer passage in those three cases is hardly sufficient to justify the conclusion that the immunity was a humoral one. Attempts were made to hyperimmunize some animals in order to repeat this experiment, but it was found that unless a period of 11 days elapsed between the first two doses the virus was liable to spread to the central nervous system. These experiments are still incomplete.

Attempts at demonstrating the presence of antibodies in the brain of immunized animals.—In the case of extracts or emulsions of the brain of immunized animals, not only were similar results obtained, that is to say, complete failure to neutralize the virus *in vitro* or to produce a state of passive immunity in new animals, but a paradoxical result was even obtained. Portions of immune brain and of virulent brain were thoroughly ground up separately with sand and centrifuged for a few minutes in order to get rid of the coarser pieces. In the case of the immune brain a thick emulsion was used, and in the case of the virulent one a thin emulsion, which protracted the incubation period in the control animal to an appreciable extent. Equal quantities of the two emulsions were mixed together and incubated for varying lengths of time at 37° C. and injected intracerebrally into new rabbits. One control animal was used for each emulsion, and the following results were obtained:

R 214	Mixture kept in contact for 5 hours	Death in 10 days	Typical encephalitis
R 215	„ „ „ 5 „	„ 6 „	„ „
R 216	„ „ „ 1 hour	„ 5 „	„ „
R 217	„ „ „ a few minutes	„ 6 „	„ „
R 218	Virus only (in same dose as above)	„ 15 „	„ „
R 225	Immune brain only (concentrated)	Recovery	

It will be noticed that with such a high dilution of the virus that it took 15 days to kill the control animal, that period was reduced to 5 or 6 days in three cases and to 10 days in one by the addition of immune brain to the virus, irrespectively of the time that the two were left in contact prior to the inoculation. A concentrated dose of the immune brain by itself produced no illness and no immunity, as the animal succumbed in 6 days to a subsequent intracerebral injection of the passage virus. The only inference which can be drawn from these experiments is that instead of the brain of an immunized animal being capable of neutralizing the virus, it increases the virulence of the latter by reducing the incubation period considerably, presumably as the result of the presence in the brain of the immunized animals of a substance which appears to act like an aggressin. In all the experiments just quoted the immune brain had been kept for a fortnight in glycerin prior to use, except in the case of R 214, where the freshly removed brain was used, and where the passage was not reduced as much as in the other cases. It is therefore possible that the aggressin-like substance does not exert its fullest effect in the fresh condition. It is interesting to recall here that Levaditi and Harvier (1920[2]) found evidence of a similar activating substance in the blood-serum of patients who had recently fallen victims to encephalitis lethargica.

It may be mentioned that an intravenous injection of an emulsion of immune brain into an animal which has just received an intracerebral inoculation of the virus, exerts no influence whatsoever on the course of the resulting disease.

Levaditi (1922, p. 216) and his co-workers had already adduced evidence to show that immunity to the virus of herpes is not a humoral one, and their conclusion receives confirmation from the experiments just quoted—at any rate in the case of animals immunized with single doses of the virus. A further

deduction seems to be justified, namely, that cerebral immunity must necessarily be essentially of a cellular nature. The question therefore arises as to whether all the cells, essential or otherwise, or groups of such cells, share in that immunity in organs like the brain. The point may be of importance, because it might account for the discrete areas of inflammation one usually observes in the brain of fatal cases of encephalitis lethargica, instead of the uniform distribution of these lesions, which one might expect in that disease, assuming that the two viruses are at least closely related.

Some interesting observations were made in the course of these immunity experiments, which appear to throw some light on the relationship between the viruses of herpes and encephalitis lethargica. Twelve immunized animals received intracerebral inoculations of the passage virus at intervals of 4 to 5 weeks in order to determine the duration of their immunity. It was found that in every case a time came when that immunity disappeared and the animal succumbed to an intracerebral inoculation of the virus. The repeated inoculations into the brain do not prolong the immunity by hyperimmunizing the animal. Death occurred in from 5 to 30 days after the final inoculation, and in 10 out of the 12 cases the typical syndrome of a herpetic encephalitis was absent. The animal instead went off its food and lay huddled up in its cage in a lethargic state. Head-nodding and some photophobia were often noticed, but lethargy and not excitement was the most prominent symptom. In 2 cases the immunity disappeared suddenly and the typical syndrome of a herpetic encephalitis with considerable excitement was present, and the virus was easily recovered from their brains after death. In the 10 animals which had shown the atypical symptoms mentioned above, the histological lesions varied a good deal. In some a very obvious encephalitis of the usual type was present. Some others showed a mild encephalitis with a patchy distribution, and consisting of a single row of lymphocytes in the perivascular spaces. The latter recalled the type of lesion sometimes found in the basal ganglia of fatal cases of the so-called post-encephalitic syndrome in man—that is to say, lesions of a subacute or chronic encephalitis. In yet a few others the lesions were hardly recognizable.

As it had been observed that the virus could not be recovered from the brains of these animals, 3 were selected where their immunity resulted from a skin, a cerebral and a corneal inoculation respectively, and where the histological lesions in the brain were very prominent in one and much milder in the other two. Further passages were attempted. Seven new animals were inoculated intracerebrally with emulsions of the brains of these selected cases. Four recovered and 3 died in 18 to 32 days with symptoms somewhat similar to those which had characterized the final illness of the 10 animals previously referred to. Lethargy was again the most prominent feature in these cases, and the typical syndrome of a herpetic encephalitis was always lacking. No virus capable of transmission in series was isolated from these cases. The percentage of deaths for these small figures works out at 43.

If we review the results of intracerebral inoculations of emulsions of brain from fatal cases of encephalitis lethargica, a close parallelism becomes apparent between rabbits partially immunized against the virus of herpes and cases of encephalitis lethargica in man.

Emulsions of brain from 14 cases of encephalitis lethargica were injected into the brain of 52 rabbits. Of these 14 died within 48 hours of a septic meningitis, cerebral hæmorrhage, etc. Of the 38 which recovered from the operation 22 showed no symptoms of any kind and survived, and 16 died in 5 to 30 days. The percentage of deaths was therefore 42.

Lethargy was the most prominent symptom present in the fatal cases. The animals ate very little, and lay huddled up in a drowsy condition in their cage. Every now and again they would shut their eyes, and their head would drop slowly as if they were going to sleep; but before the head touched the floor of the cage the animal woke up with a start to repeat the same proceeding over again. Photophobia and slight restlessness were noticed occasionally, the animal hiding its head in each corner of the cage in succession, but convulsions were never observed. Histologically the lesions were usually poor. The evidence of an encephalitis consisted of the occasional presence of a single row of lymphocytes in the perivascular spaces at fairly wide intervals. Further passages were attempted in 18 animals, 6 of which died within 1 month with symptoms somewhat similar to those present in the fatal cases of the original passage. The histological lesions were barely recognizable, and a fixed virus capable of transmission was never obtained.

The results of intracerebral inoculations of emulsions of the brains of fatal cases of encephalitis lethargica are, therefore, identical with those obtained in animals which are partially immune to the virus of herpes, and which succumb to an intracerebral inoculation of that virus. In both cases there is an inconstant incubation period varying from a few days to one month. A fatal result is obtained in the same proportion of cases, the symptoms of the terminal illness and the histological lesions are identical, and passages in series leading to the recovery of a virus with fixed characters prove unsuccessful. A further point of resemblance is found in the fact that on a few rare occasions a virus indistinguishable from that of herpes has been recovered from the brains of fatal cases of encephalitis lethargica (Levaditi and Harvier, 1920[3]; Doerr and Berger, 1922, etc.). As pointed out above, the virus of herpes can occasionally be recovered from the brain of animals which are only partially immunized against it, and whose immunity fails suddenly and completely in the presence of a fresh invasion of that virus.

If herpes febrilis and encephalitis lethargica are different manifestations of an infection by one and the same virus, the possible development of a partial cellular immunity of the central nervous system in infected individuals may account for the innumerable failures which have attended the attempts at infecting in series laboratory animals by inoculations of material from fatal cases of the disease.

One may therefore conclude that the ultimate fate of the virus on reaching the central nervous system of patients suffering from encephalitis lethargica may be one of three:

(1) A local cellular immunity may result, which is capable of overcoming the infection absolutely, with complete recovery of the patient.

(2) A state of cellular immunity develops which can only keep in check, but not neutralize completely, the virus, and leads to a chronic infection, the virus being too attenuated to be capable of transmission to susceptible animals.

(3) No immunity develops and the virus can be recovered from the central nervous system, the last being the least common of the three.

CONCLUSIONS.

(1) Cerebral immunity to the virus of herpes is best attained by intradermal inoculations of the neurotropic virus, and is fully established by the 11th day.

(2) Although cerebral immunity results from inoculations of the neurotropic virus into scarified skin, an encephalitis may result if the scarification is at all a deep one.

(3) The average period for a state of active immunity is three months, and the conclusion is reached that this immunity is essentially of a cellular nature.

(4) The state of active immunity is not prolonged by repeated intracerebral inoculations of the passage virus.

(5) The majority of immunized animals which finally succumb to an intracerebral inoculation of the passage virus show symptoms recalling those of encephalitis lethargica in man, and the virus cannot be recovered from their brain.

(6) Experimental and histological investigation of the brain of immune animals which finally succumb to an intracerebral inoculation of the virus of herpes, yield results which are identical with those obtained from inoculations of emulsions of brain from cases of encephalitis lethargica.

I wish to record my thanks to Dr. P. P. Laidlaw for much help, particularly in the preparation of this paper, and to Dr. Da Fano for many histological investigations.

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THE *IN-VITRO* CULTIVATION OF TISSUES WITH REFERENCE TO THE PRODUCTION OF CANCER BY MEANS OF RADIUM AND X-RAYS.

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THE pioneers of the *in-vitro* cultivation of tissues dealt with embryonic tissues on account of the difficulty of obtaining growth with adult tissues. Though adult tissues can now be cultivated, it is still found more difficult to cultivate the tissues of old than of young animals. Age is not, however, the factor which determines these differences. Both animal- and plant-cells can multiply, it would seem, indefinitely and do not fail through senescence. The cancer-cell does not lose its power to grow, though propagated for years by grafting from animal to animal. The repair and regeneration of lost parts shows that the cells of adults have not lost the power to grow. In most plants and in many animals, for instance fishes, growth proceeds throughout life, being largely dependent on food supply.

Drew (1923) has shown that once the conditions which hold adult cells in check are broken down, *in-vitro* growth occurs as vigorously as with embryonic or young tissues.

In the following experiment it is seen that a growth-inhibiting factor can be extracted from old tissues, which will inhibit the *in-vitro* growth of young cells.

Experimental details.—The kidneys of rats of weights 20, 80, 140, 250 gm. were emulsified and extracted with an equal volume of Locke's solution at 0° C. The kidney of a rat of 80 gm. was used for cultivation. The kidney extracts, embryo extracts and an extract of Jensen's rat sarcoma were made according to the method of Drew (1922), to whom I am indebted for help and advice in technical details. The following sets of cultures were prepared:

TABLE I.

I.	Plasma (3 parts)	+	embryo extract (2 parts)	+	Locke's solution (1 part)		+	kidney fragment.
II.	Ditto	+	Ditto	+	kidney ext. of 20 gm. rat (1 part)		+	,,
III.	,,	+	,,	+	Ditto	80 ,,	+	,,
IV.	,,	+	,,	+	,,	140 ,,	+	,,
V.	,,	+	,,	+	,,	250 ,,	+	,,
VI.	,,	+	,,	+	J.R.S. extract (1 part)		+	,,

Ten cultures were made of each set and were incubated at body-temperature for three days, when they were fixed and stained. The number of extruded cells (E.c.) and of mitoses (M.) were then counted, with the results shown below:

TABLE II.

	Locke.		20 gm.		80 gm.		140 gm.		250 gm.		J.R.S.	
	E.c.	M.	E.c.	M.	E.c.	M.	E.c.	M.	E.c.	M.	E.c.	M.
1.	1088	27	1074	27	246	1	103	1	—	—	712	13
2.	791	18	398	11	194	1	—	—	—	—	681	10
3.	779	11	335	8	37	0	—	—	—	—	34	0
4.	755	14	180	3	18	0	—	—	—	—	15	0
5.	484	7	160	2	—	—	—	—	—	—	—	—
6.	358	3	140	1	—	—	—	—	—	—	—	—
7.	Broken slide		92	1	—	—	—	—	—	—	—	—
8.	—	—	—	—	—	—	—	—	—	—	—	—
9.	—	—	—	—	—	—	—	—	—	—	—	—
10.	—	—	—	—	—	—	—	—	—	—	—	—
Totals	4255	80	2379	53	495	2	103	1	0	0	1442	23
Mitoses	1 in 53		1 in 45		1 in 247		1 in 103		0 in 0		1 in 63	

From the above results it is concluded that a growth-inhibiting factor is extracted from old tissues, but not from young, and not from Jensen's rat sarcoma. Carrel and Ebeling (1923) have shown the presence of a growth-inhibiting substance in serum which increases with age. If the standpoint be taken that adult cells have not lost the power to grow but are held in check in the adult by inhibitory factors, then the cancerous change may be merely the suppression or exhaustion of the growth-inhibiting factors, which allow adult cells to indulge in their natural tendency to grow and multiply; and there may be no need to postulate a growth-promoting factor. Experiments have thus been made to discover whether or no radium and X-rays, which both cause cancer, destroy the growth-inhibiting factor of adult tissues.

As a preliminary to the experiments with radiation, the effect of autolysis at 35° C. was investigated, for the reason that Drew (1923) has shown that a growth-promoting factor can be extracted from tissues thus treated.

Experimental details.—The kidneys of a 100 gm. rat were minced; the emulsion divided, one part kept at 0° C., the other at 35° C. After an hour extracts were made with Locke's solution. The kidney of a 125 gm. rat was used for cultivation. The following sets of cultures were made:

TABLE III.

A.	Rat plasma (3 parts)	+	rat embryo extract (2 parts)	+	Locke's solution (1 part)	+	kidney fragment.
B.	Ditto	+	Ditto	+	0° C. kidney ext. (1 part)	+	,,
C.	,,	+	,,	+	35° C. kidney ext. (1 part)	+	,,

Eight cultures were made of each set. They were kept at body-temperature for three days, then fixed and stained. The table gives the extruded cells (E. c.) and mitoses (M.) for each of the twenty-four cultures.

TABLE IV.

	Locke's solution. A		0° KE. B		35° KE. C	
	E. c.	M.	E. c.	M.	E. c.	M.
1	621	14	291	0	508	7
2	373	19	268	3	416	9
3	361	6	110	2	406	12
4	125	5	54	0	193	3
5	38	1	28	0	28	0
6	12	0	—	—	—	—
7	—	—	—	—	—	—
8	Infected		—	—	—	—
Totals	1530	45	751	5	1551	31
Mitoses	1 in 34		1 in 150		1 in 50	

A second experiment was made, using a 220 gm. rat for the kidney extracts and a kidney from an 80 gm. rat for cultivation. The same three sets of cultures were made. The following results were obtained:

TABLE V.

	Locke's solution.		0° KE.		35° KE.	
	E. c.	M.	E. c.	M.	E. c.	M.
1	417	8	504	7	160	3
2	251	10	286	3	124	3
3	206	6	126	1	108	2
4	118	4	93	0	76	2
5	112	0	67	0	72	1
6	109	0	34	0	60	1
7	69	0	—	—	—	—
8	—	—	—	—	—	—
9	—	—	Infected		—	—
Totals	1282	28	1110	11	600	12
Mitoses	1 in 46		1 in 101		1 in 50	

The two experiments show a diminished rate of cell division as measured by the number of mitoses in the 0° KE as compared with the heated kidney extract. A similar effect is more clearly seen in the *in-vitro* experiments about to be described.

RADIATION EXPERIMENTS.

The experiments with radiation fall under two headings, *in-vitro* and *in-vivo* experiments.

Details of the in-vitro experiments.—Radium was used as the source of radiation in all these experiments. Kidney emulsion from rats of 125–200 gm. was used. A part was kept in an ice-chest, and the rest was radiated in an ice-chest between sheets of mica (0·05 mm. thick) sealed with vaseline. The

thickness of the emulsion was in the neighbourhood of 1 mm. Two radium applicators 4 cm.^{2} (110 mgm. $RaBr_2$, $2H_2O$) screened by 0·12 mm. silver were used, being placed one on either side of the mica cell. The time of radiation varied from two hours to six days. In a few cases some of the kidney emulsion was incubated at 35° C. for one to two hours.

The tissue cultures were made in the following way: Embryo extract (EE) was mixed with kidney extract (KE) [whether kept on ice (0° KE), incubated at 35° C. (35° KE), or radiated in an ice-chest (RaKE)] in a glass cell in known proportions, the drop method being used to measure the amounts. Fragments of adult kidney were next placed on cover-glasses and a drop of the EE and KE mixture was added. Lastly, an equal volume (drop method) of plasma was added and stirred in. After coagulation the cultures were sealed on to hollow-ground glass slides and incubated for three days at body-temperature.

The following is a sample of a single experiment in which the exposure to radium was for eighteen hours. The ingredients of the cultures are given in Table VI.

TABLE VI.

I.	EE 2 parts	+ 0° KE 1 part	+ plasma 3 parts	+ fragment of adult kidney.		
II.	EE 2 „	+ RaKE 1 „	+ „ 3 „	+ „	„	
III.	EE 2 „	+ RaKE $\frac{5}{7}$ „	+ Locke's solution $\frac{2}{7}$ part	+ plasma 3 parts	+ ditto.	
IV.	EE 2 „	+ RaKE $\frac{5}{9}$ „	+ „ „	$\frac{4}{9}$ „ + „	„	+ „

Series I served as a control. 6 cultures were made.
„ II was an experimental series of dilution 1 in 6. 7 were made.
„ III „ „ „ „ 1 in 8·4. 6 „ „
„ IV „ „ „ „ 1 in 10·4. 6 „ „

After three days the slides were stained and examined under the microscope. They were marked—no extrusion = 0; slight extrusion = +; extrusion = ++; and good extrusion = +++. The nature of the slide was unknown at the time of marking. The marking requires further explanation, as the better method of counting the extruded cells and mitotic figures had not then been adopted. Specimens showing a complete ring of extruded cells, the numbers amounting to many hundreds, were marked +++. When the ring was incomplete, the numbers of cells being several hundred, the marking was ++. When only one or two points of extrusion were present, the cells being numbered in tens rather than hundreds, the marking was +. The following table gives the results obtained in the experiment under description:

TABLE VII.

1. Control.	II. Exp. 1 in 6.	III. Exp. 1 in 8·4.	IV. Exp. 1 in 10·4.
1 ++	1 +++	1 +++	1 ++
2 ++	2 +++	2 ++	2 ++
3 +	3 ++	3 ++	3 ++
4 0	4 ++	4 +	4 +
5 0	5 ++	5 0	5 0
6 0	6 0	6 0	6 infected
	7 0		
Average $\frac{5}{6}$ = ·83	$\frac{12}{7}$ = 1·71	$\frac{8}{6}$ = 1·33	$\frac{7}{5}$ = 1·40

The average figures thus obtained serve to give a broad view of the results and are used in the following Table VIII, which sets out the whole of the experimental findings. For instance, in series E are seen the figures 0·83, 1·71, 1·33, 1·40, obtained in the experiment given in detail (see Table VII).

TABLE VIII.

	Controls. Dilution		Time of radiation.	Radiated. Dilution			
	0° C. 1 in 6.	35° C. 1 in 6.		1 in 6.	1 in 8·4.	1 in 10·4.	1 in 22·8.
A	0·20		2 hours	0·66	0·29		
B	0·20		4 „	1·23	0·66	0·00	
C	0·00		8 „	0·83	1·57	0·33	0·65
D	0·00	1·66	17 „	1·62			
E	0·83		18 „	1·71	1·33	1·40	
F	0·80		2 days	1·83		2·66	
G	0·65		3·5 „	1·60		1·30	2·37
H	0·50		6 „	2·00		0·30	
I		1·60	6 „	1·60	1·30	0·16	
	$\frac{14}{68}$	$\frac{21}{28}$	—	$\frac{47}{75}$	$\frac{38}{58}$	$\frac{31}{78}$	$\frac{22}{25}$
	0·21	0·75	—	0·63	0·65	0·41	0·78

A second method of estimating the results was used, which perhaps will be more convincing to those unacquainted with *in vitro* cultures. Instead of estimating the amount of extrusion, the slides were divided into those showing no extrusion and those showing extrusion. The results are given below the columns in the table; for instance, the fraction under the first column means that out of a total of 68 cultures 14 presented extrusion. The corresponding decimals show that on the whole the radiated showed three times as much extrusion as the controls. The same applies to the heated kidney emulsion, column No. 2. It is to be noted that the controls to the longer periods of radiation present some extrusion. This is probably due to the long exposure of the cells in the ice-chest at 6–8° C., not 0° C., allowing of some autolysis, and thus the disappearance of the inhibitory factor in the control.

The experimental error in the *in-vitro* cultivation of tissues is high, so that detailed conclusions are not permissible. For instance, turning to Table II, under "20 gm." we see that the number of extruded cells varies from 1074 to 0, and of mitoses from 27 to 0, in ten cultures. The question arises, given such wide variations, what difference in number of extruded cells and of mitosis represents a difference in the controlled experimental conditions and not a difference in those variables out of the experimenter's control. It is clear that the protocols set out in Table II do not establish any difference between "Locke," "20 gm." and "J. R. S." Whether the "140 gm." and "250 gm." results are likewise due to uncontrolled variables is entirely a matter of judgment, and the same applies to all the experiments with *in-vitro* cultures detailed in this paper. *In-vitro* cultivation is at present not open to statistical investigation. It would seem that at present the *in-vitro* cultivation

of tissues is more useful in the suggestion of biological effects to be confirmed in other ways than in their direct establishment, and this applies to the whole of the work here detailed.

Table VIII shows that the growth-inhibiting factor present in kidney extracts of adult animals is destroyed by exposure to radium, just as does exposure to 35° C. for 1 or 2 hours. Further, it appears that comparatively small doses (4 hours) are sufficient, and that large doses (6 days) do not greatly increase the effect. In these experiments extrusion and not mitoses was measured, and therefore the use of the word "growth" in the conclusion might appear unjustified.

Under a few circumstances it is possible to have extrusion without mitosis, as when radiated tissues are cultivated (Price-Jones and Mottram, 1914), but in most cases the two go hand-in-hand. Moreover, as will be seen, in the *in-vivo* experiments this criticism does not hold.

Details of the in-vivo *experiments.*—As sources of radiation both X-rays and radium were used. The best results were obtained when the kidney was extracted immediately after the radiation was stopped. These experiments will therefore be given in greater detail than when various intervals of time were permitted to elapse between the radiation and the making of the kidney extract.

Thirty minutes' X-rays was given to a rat of 120 gm., loose in a box. The dose of X-rays was controlled by means of an ionization chamber at 220 volts connected with a galvanometer standardized against a standard cell (for particulars see Appendix).

Immediately after the X-rays' exposure an ice-cold extract of the kidneys was made with Locke's solution. A control rat of 125 gm. was similarly dealt with. A piece of the control rat's kidney was kept for cultivation. The following sets of cultures were made:

I. Plasma 3 parts + embryo extract 2 parts + Locke's solution 1 part + kidney fragment.
II. „ „ + „ „ + Control KE 1 „ + „
III. „ „ + „ „ + X-ray KE 1 „ + „

The cultures were incubated for 3 days as usual, then fixed and stained, and the following counts made:

TABLE IX.

	Locke's solution.		Control KE.		X-ray KE.	
	E. c.	M.	E. c.	M.	E. c.	M.
1.	303	3	86	0	186	11
2.	148	2	64	0	169	4
3.	138	10	3	0	153	7
4.	119	9	—	—	122	3
5.	95	0	—	—	104	1
6.	90	0	—	—	92	2
7.	—	—	—	—	76	0
8.	—	—	—	—	39	2
9.	—	—	—	—	19	0
10.	—	—	—	—	—	—
Total	893	24	153	0	960	30
Mitoses	1 in 37		0		1 in 32	

In the next experiment radium (110 mgm. screen 0·12 mm. silver + thin rubber) was applied to the anterior surface of the kidney for 3 hours, the abdomen being opened under urethan and ether. The abdomen of the control rat was likewise opened for 3 hours. Both animals weighed 250 gm. Immediately the radium was removed the kidney was removed and extracted in the usual way, likewise the control kidney. The kidney from a 60 gm. rat was used for cultivation.

The usual cultures were made, and finally stained and counted.

TABLE X.

	Locke's solution.		Control KE.		Radium KE.	
	E. c.	M.	E. c.	M.	E. c.	M.
	439	13	401	3	396	8
	416	4	56	1	367	4
	410	10	41	0	237	5
	345	12	24	0	238	2
	331	11	17	0	234	3
	330	4	—	—	184	3
	270	3	—	—	—	—
	264	6	—	—	—	—
	214	4	—	—	—	—
	174	2	—	—	—	—
	146	3	—	—	—	—
	8	0	—	—	—	—
Totals	3347	72	539	4	1656	25
Mitoses	1	46	1	135	1	66

TABLE XI.

	Locke's solution.		Control KE.		Radium KE.	
	E. c.	M.	E. c.	M.	E. c.	M.
	984	30	432	1	592	27
	738	15	306	6	544	16
	673	19	248	1	327	5
	551	18	186	0	326	5
	498	19	173	0	284	6
	380	5	163	1	122	2
	322	1	163	0	77	1
	305	0	25	0	—	—
	182	0	21	0	—	—
	125	1	—	—	—	—
	82	0	—	—	—	—
	32	0	—	—	—	—
Totals	4872	108	1717	9	2272	62
Mitoses	1 in 45		1 in 191		1 in 37	

In the third set of cultures radium was applied to the kidney from the back by removing a piece of skin and radiating through the thin muscles of the loin.

of tissues is more useful in the suggestion of biological effects to be confirmed in other ways than in their direct establishment, and this applies to the whole of the work here detailed.

Table VIII shows that the growth-inhibiting factor present in kidney extracts of adult animals is destroyed by exposure to radium, just as does exposure to 35° C. for 1 or 2 hours. Further, it appears that comparatively small doses (4 hours) are sufficient, and that large doses (6 days) do not greatly increase the effect. In these experiments extrusion and not mitoses was measured, and therefore the use of the word "growth" in the conclusion might appear unjustified.

Under a few circumstances it is possible to have extrusion without mitosis, as when radiated tissues are cultivated (Price-Jones and Mottram, 1914), but in most cases the two go hand-in-hand. Moreover, as will be seen, in the *in-vivo* experiments this criticism does not hold.

Details of the in-vivo *experiments.*—As sources of radiation both X-rays and radium were used. The best results were obtained when the kidney was extracted immediately after the radiation was stopped. These experiments will therefore be given in greater detail than when various intervals of time were permitted to elapse between the radiation and the making of the kidney extract.

Thirty minutes' X-rays was given to a rat of 120 gm., loose in a box. The dose of X-rays was controlled by means of an ionization chamber at 220 volts connected with a galvanometer standardized against a standard cell (for particulars see Appendix).

Immediately after the X-rays' exposure an ice-cold extract of the kidneys was made with Locke's solution. A control rat of 125 gm. was similarly dealt with. A piece of the control rat's kidney was kept for cultivation. The following sets of cultures were made:

I. Plasma 3 parts + embryo extract 2 parts + Locke's solution 1 part + kidney fragment.
II. " " + " " + Control KE 1 " + "
III. " " + " " + X-ray KE 1 " + "

The cultures were incubated for 3 days as usual, then fixed and stained, and the following counts made:

TABLE IX.

	Locke's solution.		Control KE.		X-ray KE.	
	E. c.	M.	E. c.	M.	E. c.	M.
1.	303	3	86	0	186	11
2.	148	2	64	0	169	4
3.	138	10	3	0	153	7
4.	119	9	—	—	122	3
5.	95	0	—	—	104	1
6.	90	0	—	—	92	2
7.	—	—	—	—	76	0
8.	—	—	—	—	39	2
9.	—	—	—	—	19	0
10.	—	—	—	—	—	—
Total	893	24	153	0	960	30
Mitoses	1 in 37		0		1 in 32	

In the next experiment radium (110 mgm. screen 0·12 mm. silver + thin rubber) was applied to the anterior surface of the kidney for 3 hours, the abdomen being opened under urethan and ether. The abdomen of the control rat was likewise opened for 3 hours. Both animals weighed 250 gm. Immediately the radium was removed the kidney was removed and extracted in the usual way, likewise the control kidney. The kidney from a 60 gm. rat was used for cultivation.

The usual cultures were made, and finally stained and counted.

TABLE X.

Locke's solution.		Control KE.		Radium KE.	
E. c.	M.	E. c.	M.	E. c.	M.
439	13	401	3	396	8
416	4	56	1	367	4
410	10	41	0	237	5
345	12	24	0	238	2
331	11	17	0	234	3
330	4	—	—	184	3
270	3	—	—	—	—
264	6	—	—	—	—
214	4	—	—	—	—
174	2	—	—	—	—
146	3	—	—	—	—
8	0	—	—	—	—
Totals 3347	72	539	4	1656	25
Mitoses 1	46	1	135	1	66

TABLE XI.

Locke's solution.		Control KE.		Radium KE.	
E. c.	M.	E. c.	M.	E. c.	M.
984	30	432	1	592	27
738	15	306	6	544	16
673	19	248	1	327	5
551	18	186	0	326	5
498	19	173	0	284	6
380	5	163	1	122	2
322	1	163	0	77	1
305	0	25	0	—	—
182	0	21	0	—	—
125	1	—	—	—	—
82	0	—	—	—	—
32	0	—	—	—	—
Totals 4872	108	1717	9	2272	62
Mitoses 1 in 45		1 in 191		1 in 37	

In the third set of cultures radium was applied to the kidney from the back by removing a piece of skin and radiating through the thin muscles of the loin.

The control rat, similarly, had a piece of skin removed.

The exposure was for 3¼ hours. The rats weighed 245 gm.; the kidney for cultivation was of a 60-gm. rat. The extracts were made directly the radiation was finished.

The figures given in Table XI resulted.

A fourth set, using X-rays to the whole animal for 35 minutes as in the first set, gave the following totals:

TABLE XII.

Locke's solution.	Control KE.	X-ray KE.
1282 28	1043 11	1551 25
1 in 46	1 in 95	1 in 62

Reviewing these four sets, it is seen that the number of cells extruded is much the fewest in the control cultures and also that the percentage of mitoses is greatly reduced. It is thus concluded that the inhibitory factor present in the normal kidney extract of the adult rat is destroyed by the *in-vivo* radiation of the kidney.

Experiments in which the kidney extract was not made immediately after the termination of the radiation will now be briefly dealt with.

TABLE XIII.

(1) 24 *Hours after* 30 *Minutes' X-rays.*

	Control KE.	X-ray KE.
Totals	3679 31	8156 139
Mitoses	1 in 118	1 in 59

(2) 2 *Days after* 30 *Minutes' X-rays.*

	Control KE.	X-ray KE.
Totals	321 2	664 9
Mitoses	1 in 160	1 in 73

(3) 10 *Days after* 40 *Minutes' X-rays.*

	Locke.	Control KE.	X-ray KE.
Totals	962 20	181 1	525 5
Mitoses	1 in 48	1 in 181	1 in 105

Here it is seen that whereas there is some effect after 24 hours (in this experiment rats of 100 gm. were used, which may account for the considerable growth in the control), there is very little after 2 days and none after 10 days. It appears that this effect of radiation is soon recovered from, though further experiments are required to define exactly the recovery-rate.

GENERAL DISCUSSION.

We may look upon cancer as a disturbance of the nice balance of growth and the harmony of repair whereby the proper dimensions of the various organs of the body are maintained from birth onwards.

Very little is known as to how this balance is maintained. It is known that the growth-changes in the metamorphosis of amphibians is under the

control of the thyroid secretion, and that maleness and femaleness, potentially present in the cells of many animals throughout life, is only expressed under suitable hormone action. For the most part the growth-rate of different organs during development is approximately equal; but cases are known where the growth-rate of an organ is greater than that of the rest of the body, as in the chela of the male fiddler crab, or smaller, as is the case in many degenerate adult organs which function only in early life. Nothing is known as to how these various growth-rates are controlled, or how, for instance, the skin and mucous membranes are continually being renewed in a harmonious manner by the growth and division of cells. It is to be observed that cancer occurs most commonly in tissues where cell-division is continuous throughout life, where, in fact, conditions unfavourable to growth are not strong. By exposing the skin to radium or X-rays the normal balance is disturbed, so that either simple overgrowths occur in the form of warts or malignant epitheliomas.

The experiments here described suggest that this is brought about by the destruction of growth-inhibiting factors rather than by the production of growth-stimulating factors.

Whether the results bear any relation to what occurs in living tissues or whether they are only a peculiar property of *in-vitro* cultivation, further work alone can show.

It has been the custom to speak of, if not to look for, a stimulating factor in the cause of cancer; the present considerations suggest as an alternative that cells have a natural tendency to grow and divide which in normal conditions is held in check by varying inhibitory factors.

From the point of view of radio-biology some discussion is required. Hitherto the observed biological effects of radiation have shown a latent period between their manifestation and the application of the radiation. The conclusion has been drawn that immediate changes must occur, but nothing has been detected, or measured, until after a certain lapse of time, which may vary from a few hours up to many days. In these experiments a change has been detected immediately after the exposure has been made, which moreover appears to be greatest at this time and to decrease as time passes. The failure to detect immediate effects has stood in the way of the investigator anxious to discover how radiations produce their biological effects.

Though these investigations do not appear to be of any help in this respect, they form, at any rate, a starting-point for further research into the mysteries of the latent period.

CONCLUSIONS.

(1) Extracts made at 0° C. from the kidneys of adult rats inhibit the *in-vitro* growth of kidney-cells.

(2) Exposure of emulsified kidneys to 35° C. for one or two hours before the extract is made destroys the inhibitory factor.

(3) Exposure of emulsified kidneys *in-vitro* to radium has a like action.

(4) Exposure of kidneys *in-vivo* to radium or X-rays likewise destroys the inhibitory factor.

(5) This effect of radiation is immediate, and grows less if an interval of time be allowed to elapse between the radiation to the kidney and the making of the extract.

The radium used in these researches was on loan from the Medical Research Council.

Appendix: X-ray Measurements.

Use was made of an ionization chamber at 220 volts connected to a galvanometer. The ionization chamber placed between the anti-cathode and the animal measured 8 × 6 × 12 in.; distance from anti-cathode, 12 in.; sides of chamber, $\frac{5}{100}$ mm. aluminium; wood of animal box, 3 mm.; depth of animal box, 8 in.; distance of animal, 16 in.

Galvanometer against a standard cell gave 0·085 micro-amps. per scale division.

X-rays giving 4·2 divisions of galvanometer and running for 1·2 hours give a dose of one rad.

The following were the dial readings: Heating current, 4·2 amps.; primary current, 7 amps.; spark-gap, 18 cm. One rad is the exposure required to just kill Jensen's rat sarcoma *in-vitro*.

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TETANUS.—I. ISOLATION, MORPHOLOGY AND CULTURAL REACTIONS OF *B. TETANI*.

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THE following account of the bacteriology of *B. tetani* arises out of an observation which permits of the examination for the first time of a large number of pure strains.

The fact that under suitable conditions *B. tetani* has a tendency to grow as a spreading film on the surface of a medium has been noted by several, but it has not been realized that this character of spreading growth is much more pronounced in the case of *B. tetani* than with other anaerobes.

If the water of condensation of a sloped tube of agar be inoculated with *B. tetani*, in the course of one or two days the growth will have spread to the apex of the surface. If the strain so inoculated be not pure the tetanus bacilli will still grow to the top, but the contaminating organisms will spread up only to a minor extent. Thus the tetanus bacilli may be transferred from the top edge of the growth to another tube and may grow there in pure culture.

This technique has been applied to a large number of samples of tetanic material. Many pure cultures of *B. tetani* have been obtained and their characters are recorded here. It may be stated at once that the strains identified in this paper as *B. tetani* are so identified from their agglutination with tetanus sera, and that this identification is supported by morphology and cultural tests although a considerable number are found to be non-toxic.

Little apology is required for discussing the characters of *B. tetani*, because the accounts of these are obviously conflicting in the literature. To refer only to the morphology of the sporing bacillus, this is not always described as "drum-stick." Thus Heller (1922) says: "Occasionally a strain is found that repeatedly forms spores centrally." Dean and Mouat (1916) also found that some of the spores "were oval in shape and were either in the centre of the bacillus or . . . . ." German writers now describe the bacillus as "tennisschlägerförmig" (Zeissler, 1923; Buzello and Rahmel, 1924), which can only imply that the spore is oval. Indeed, in the first description of the spore by Flügge (1886), in whose laboratory Nicolaier worked, it is said to be oval.

Furthermore the characters of the bacillus as at present described are based upon a study of a small number of strains which are not necessarily all different. Thus among recent authors Adamson (1919–20) described 3 strains; Hall (1922), 7; McIntosh (1917), 8; Heller (1922), 8; Zeissler (1923), 12; and of these it would appear that only 4 were new in the sense of having been isolated by the authors quoted. Tenbroeck and Bauer (1922) isolated over 30 pure cultures but gave no description of them, while the accounts of a few other authors leave grave doubt of the purity of their strains. This paucity of new cultures is, of course, due to the difficulty of isolating *B. tetani* for examination, not to any lack of tetanic material. Zeissler (1923) says: "For instance in tetanus neonatorum up to now pure cultures of tetanus bacilli have been obtained on only two occasions, once by Kitasato in 1891, and once by the author and Käckell in 1921. In the intervening thirty years some fifty papers have appeared on this subject and in none could pure cultures be claimed." This statement may, perhaps, give a false idea of the difficulty, but it may be accepted as an indication that some difficulty exists.

In the present work a strain is claimed to be pure when its characters are constant and do not deviate essentially from those of other members of the same group.

TECHNIQUE.

The media used for the cultivation of *B. tetani* throughout this investigation have been the ordinary media used in this laboratory for general purposes for years. Such media as those known as "cooked meat" and "brain-agar" have been expressly avoided, because in our experience their sterility is extremely difficult to establish, and they can account for some of the impurities which are said to appear in old stock cultures of *B. tetani*.

In general, the exact constitution of the medium is possibly of little importance, but at the same time, the cultural and other characters of all strains of tetanus bacilli cannot be disclosed satisfactorily with a poor medium. Meat broth is a necessary basis, and the addition of digested blood or laked blood is frequently desirable, the latter especially with gelatine and coagulated serum cultures.

The routine solid medium has been "peptic blood-agar" (Fildes, 1920[2]), which grows *B. tetani* in a manner similar to ordinary agar but more copiously. Sloped tubes should be kept before use until the apex of the slope shows a tendency to drying, otherwise the characteristic edge of the film-growth will not be seen. Liquid media were enriched with 2 per cent. of the peptic digest, while gelatine and coagulated serum had added ½ per cent. ox blood laked with an equal part of distilled water and preserved with chloroform. Liquid media and deep agar tubes were always boiled and shaken before inoculation and placed rapidly under anaerobic conditions. Inoculation of liquid media was made with two drops of a suspension from a slope culture. No glucose or other reducing agent was added to any medium. Gelatine was incubated at 37° C., and then tested for liquefaction in cold water.

Anaerobic conditions were obtained with the various applications of McIntosh and Fildes' anaerobic methods elaborated from the original suggestion of Laidlaw (McIntosh and Fildes'

1916; Fildes, 1917; Fildes and McIntosh, 1921). When it was required to examine tubes from day to day the single-tube method was used (Fildes, 1917); for large numbers of tubes or plates the electrically-heated jar (Fildes and McIntosh, 1921).

Certain writers of encyclopædic compilations are apt to depreciate these methods apparently without having tried them (Zeissler, 1923, and others). They may be referred to the writings of Colebrook (1920), M'Leod and Gordon (1923) in this country, and of Smillie (1917), Brown (1921), Zinsser (1922), and Richardson and Dozler (1922) in America.

It would appear that none of these authors have adopted the single-tube method.

Several American workers using electrically-heated jars have given warning of the danger of explosion. We had never experienced explosions during our experience of eight years until recently. On this occasion the heating capsule was found not to have been made according to our specification, and the spark from a broken wire ignited the mixture of gases. This should not occur if the apparatus is properly made, but nevertheless, we now place a loose cover over the whole jar when the current is first turned on.

ISOLATION.

When the material to be examined contains numerous tetanus bacilli, as for instance do most soils, a small quantity may be placed directly into the condensation water of a sloped tube. Generally, however, and particularly in cases of tetanus, it is preferable to make an enriching culture. About 2 gm. of earth or fæces, or larger quantities of material from tetanic wounds, or, indeed, the whole wound excised, are placed into freshly-boiled blood-broth and incubated in air for at least 2 days, preferably 4. The blood-broth is used in this laboratory for various purposes, but any medium containing tissue will serve equally well. Spores do not form readily in the blood-broth medium, and therefore every tube, without microscopic examination, is subcultured (2 drops) to the condensation water of a sloped peptic blood-agar tube, which is then incubated anaerobically at 37°C. In 24 hours, or occasionally in 48 hours, it is found that growth has spread up the surface. Different bacteria will be recognized by the occurrence of "high-water marks" indicating the limits of their growth (Fig. 30), but above the highest, upon the apparently unaffected surface of the medium, an exceedingly fine almost structureless film will sometimes be observed with difficulty with a hand lens. This film may be practically invisible except at the top spreading edge, where it encroaches upon the drying margin of the slope. At this point a tangle of extremely fine filaments may be seen spreading up from the film, and this may prove to consist of tetanus bacilli. It should be examined and subcultured to the condensation water of another tube. This second tube will usually be found to grow a pure structureless film with filamentous edge, but if not pure a further subculture from the edge to the condensation of another tube will nearly always succeed. The only organism which up to now has been found to resist this method of elimination is *B. proteus*, but this can, of course, be removed by heat.

During the course of this work the following materials have been examined:

Material from human cases of tetanus	6 specimens	6 positive.
Human fæces (hospital patients)	200 ,,	2 ,,
Horse fæces (London stable horses)	200 ,,	34 ,,
Soil (cultivated and waste lands)	70 ,,	33 ,,

Specimens were examined from the original slope culture from any spreading films which might be present, and also usually from the mixed

growth at the bottom. In no instance, however, were drum-sticks found at the bottom of the tube without a spreading film with drum-sticks appearing at the top; thus it is probable that whatever tetanus bacilli were present in the samples, grew as films at the tops of these sloped tubes. Subcultures were also taken from films in which no spores were demonstrable after 4 days' incubation, and in this way several strains of late-sporing tetanus bacilli were obtained.

A relatively small number of bacilli also were isolated which grew in spreading films, but with an edge more or less conspicuously different from that of typical tetanus bacilli. These films had rounded smooth edges, or edges with rounded finger-like processes, or filamentous processes or outgrowths more opaque and thicker than those of tetanus. In many cases these films contained bacilli with terminal more or less round spores, not differing essentially from those of tetanus bacilli, but some of these strains fermented sugars, while none agglutinated with 5 different tetanus sera and none were toxic. From lack of evidence of relationship these strains were excluded from the group identified as tetanus bacilli (*cf.* p. 67).

THE APPEARANCE OF SURFACE CULTURES OF *B. TETANI.*

When a streak inoculation of *B. tetani* is made upon a plate or tube of peptic blood-agar (and so far as my experience goes, upon ordinary agar), colony-formation is very seldom apparent. This rule applies if the medium is normally moist, but if specially desiccated plates or tubes are used colony-formation is more common. The growth is often seen to be thicker at the point of inoculation, but it spreads as a film in all directions, visible with a hand lens for some distance, and with a $\frac{2}{3}$ in. objective to the limits of the plate. Indeed, the growth usually continues to the back of the agar, and motility may then be observed in the film between the medium and the glass. If the edge of the film can be observed under the microscope it will be observed to consist of long spreading filaments.

The most typical appearance is seen when the condensation water of a sloped tube is inoculated. In about 15 hours, varying very much with the strain, and with the activity of the growth, the edge of the film will be observed with difficulty about half way up the tube. Long curved filaments, widely separated, will be found reaching upwards; the body of the film will often give the effect of horizontal ridges or striations. The film will spread upwards roughly at the rate of 1 cm. per hour until the filaments have reached that part of the tube where a certain degree of dryness exists. At this time the body of the film may be structureless like dew on glass, but usually it shows a very characteristic appearance, which may be likened to crowded spots of salad oil on water (Fig. 1). Later it becomes obviously opaque like ground-glass. The stationary top edge of the film requires chief attention. After 17 to 24 hours' incubation the chief feature is the extremely fine threads of bacilli, which reach upwards and cease more or less on a uniform horizontal line where the medium has become dry. If the apex of the medium is narrowed and drawn out, as when the agar has run down the side of the tube in filling, the filaments tend to arrange themselves in a definite straight and

parallel course; when the top of the medium is broad, they are more irregular, recurved or tangled (Figs. 1 to 8). Some cultures produce much longer filaments than others, but not always. It is possible that toxic strains more often show long filaments. In cultures 1 to 3 days old, slight irregular nodosities are often seen in the upper part of the film below the filamentous edge, and the filaments themselves often develop on their stems minute spiral nodosities, or at their tips more opaque swellings. With increasing age of the culture, the filaments, which at first are hardly visible with a hand lens, become progressively more opaque and visible to the naked eye.

When film growths with the characters described are found in an anaerobic tube, they may be said to be practically diagnostic of tetanus. It very seldom happens that bacilli are met with which prove not to be *B. tetani*, but yet have a close resemblance to the filamentous growth of this bacillus.

THE MORPHOLOGY OF *B. TETANI*.

The limits of variation in the morphology of *B. tetani* appear to be wider than is generally supposed, even when the precaution is taken to examine only cultures growing under satisfactory conditions.

In the non-sporing condition the bacillus shows no specially characteristic feature. In a specimen stained with methylene-blue, it is seen to be a bacillus with slightly rounded ends varying in width from 0·6 μ to 0·3 μ. Its length is indeterminate from a few μ to over two diameters of the field (Figs. 9 and 10). These long filaments are to be found in the youngest surface cultures, and there is seldom any indication of division into constituent bacilli, although after 24 hours this division nearly always takes place. Those bacilli which do not form spores gradually lose their affinity for aniline dyes, and in many cases become almost completely autolyzed. The development of spores in some cultures take place sparsely, and in others freely. A strain which forms spores readily usually does so on the second day of incubation on peptic blood-agar, but on the third or fourth on media containing much unchanged protein. The feeble sporers may show only a few after 8 to 10 days' incubation.

The morphology of the spore depends upon its state of development. At first it is a slight oval enlargement of one end of the bacillus, not capable of retaining fuchsin (Figs. 14 and 15). It is next found to stain deeply with fuchsin, to acquire gradually a spherical shape and expand to the typical "drum-stick." During this process the intensity of the staining with fuchsin often becomes less. The remnants of the protoplasm of the bacillus which originally surrounded the spore vanish early except on the distal side, where a small cap often persists (Figs. 12 and 13). The outline of the spore is usually darker and more sharply cut than those of most other bacilli. They do not attain their mature size and shape without acquiring the fuchsin-retaining character, nor do they readily become free from the bacillus even in old cultures. When this does occur a fragment of the body often remains attached to the spore, and the appearance is then reminiscent of *B. sphenoides*. While the spore is developing, the body of the bacillus tends to lose its affinity for dyes, but it does not become autolyzed to the same extent as the non-sporing bacillus. It tends to remain short—about 4 diameters of the spore, but it may be 3 or 4 times longer.

The familiar description of the sporing bacillus with spherical terminal spore is undoubtedly to some extent idealistic, unless this description is retained for " mature " spores and other forms are looked upon as " immature." Some cultures certainly tend to retain the oval shape or do not attain a true sphere (Figs. 16, 17, 18), while other cultures produce bizarre or involution forms in which the spore may be obviously oval (Figs. 20, 21). The bacilli in such cultures often remain filamentous and spores are rare. These frequently retain the surrounding rim of protoplasm which stains blue, and thus appear unusually large (Fig. 19), while " dumb-bell " types with two spores may be observed (Figs. 25, 26). Sometimes associated with dumb-bell types are long bacilli in which the fuchsin-retaining material of the spore is arranged as amorphous granules along the course of the filament (Figs. 23, 24).

The pyriform swellings described by early writers are not common under ordinary conditions of growth. They are pear-shaped or oval swellings at any point in the body of the bacillus, which do not retain fuchsin, but have an unusually strong affinity for methylene blue (Figs. 22, 23). When occurring at the end of the bacillus they are hardly distinguishable from the early stage of spore development, and it is possible that they represent an aberrant and abortive form of spore formation.

The morphological picture as described above is to be found among all strains of tetanus bacilli, whether toxic or non-toxic, and also to some extent among bacilli which do not agglutinate with tetanus sera (Figs. 27, 28, 29). Nevertheless it may be said that a toxic tetanus bacillus is more commonly typical than a non-toxic, while a non-agglutinating bacillus shows the greatest abnormality. The abnormality is not, however, sufficiently constant or marked to differentiate these latter bacilli from toxic or non-toxic tetanus bacilli.

CULTURAL REACTIONS OF *B. TETANI.*

The behaviour of *B. tetani* under different conditions of medium is not described uniformly by the authors. The present account is in general agreement with that of Hall (1922) where they deal with the same points.

Broth.—Cultures in broth or in broth containing blood-derivatives frequently show no opacity until the second day. A general opacity then develops and is maintained for a long time, except in the case of certain strains. These latter in 3 or 4 days become quite clear, the growth falling to the bottom of the tube. This effect is, no doubt, related to the behaviour of the same strains in the agglutination test (*cf.* forthcoming paper).

Obvious gas-formation has not been observed in liquid cultures (Durham's tube), but occasionally a few minute bubbles may appear on shaking. Indol was detected after 7 days in all of 70 strains tested.

Agar: Shake cultures.—Agar shake cultures incubated in air, when heavily inoculated, develop a diffuse opacity below the line to which air has penetrated. In common with other anaerobes, a zone of heavy growth is often observed at or about the limit of air penetration. This limit may be demonstrated sharply by the addition of laked blood to the medium, when the aerobic zone will contain methæmoglobin and the anaerobic zone crimson reduced hæmoglobin. As a shake culture continues to develop, it will be

found, nearly always, that the growth spreads upwards from the limiting zone into the previously aerobic area, and that the methæmoglobin is reduced thereby. When a moist agar (about 0·3 per cent.) is used, growth will even be found in the condensed water upon the surface. Hall (1922) has stated that growth in the aerobic zone is indicative of impurity of culture, but it would appear that this is not necessarily the case, since it is observed with single cell isolations (National Collection of Type Cultures No. 279). It is more probable that the spread of growth is due to a reduction of the aerobic area by the actively growing bacilli. The appearance of bacilli in the condensation water is clearly due to shrinkage of the agar, or to the conveyance of the bacilli from below by capillarity.

By light inoculations into 2 per cent. agar shake cultures, single colonies may be obtained with relative difficulty. These resemble the descriptions of McIntosh (1917), Adamson (1919–20), Heller (1922) and others.

With regard to the production of gas in shake cultures, this seems to depend largely upon the concentration of the agar. Using 0·3 per cent. agar no gas is observed, but using 2 per cent. agar, heavily inoculated, a few bubbles of gas may appear and lead to a splitting of the medium. The appearance of gas-bubbles seems to depend simply upon the solubility of the gas in the medium.

When blood is added to agar for shake cultures a certain amount of blackening takes place. This is so whether laked blood or pepsin-digested blood is added. The darkening of the medium is practically confined to the limiting zone of air penetration, and thus appears as a ring, which after a number of days becomes jet black. The development of the black colour is dependent upon the penetration of air into the medium, since it does not appear at all in the anaerobic jar. In heated blood-agar the ring has at first a green tinge, as stated by M'Leod and Gordon (1923), but later this also becomes black.

Gelatine.—Growth of most strains of tetanus bacilli in gelatine at 22° C. is absent, or so feeble as to be of little value as a test. Even at 37° C. growth is poor and inconstant. If, however, about 1 per cent. of laked ox-blood is added, then growth is relatively satisfactory. A diffuse turbidity is produced with deposition after a few days.

Although the tetanus bacillus leads to a liquefaction of gelatine, as stated by all authors except Kendall, Day and Walker (1922), nevertheless this does not take place with equal facility with all strains. Whereas some will cause liquefaction in 3 or 4 days at 37° C., others will require 3 or 4 weeks or must be tested more than once. Of the 58 strains tested, 5 did not liquefy gelatine until after 3 weeks. Of these, 2 (TH 5 and TH 10) were toxic; the others were non-toxic. It is often observed that liquefaction takes place after the growth has sunk to the bottom of the tube and has presumably ceased. This would suggest that it is due to a ferment elaborated by the bacilli and continuing to operate after growth is suspended.

When liquefaction of gelatine has taken place, the production of gas can often be demonstrated by shaking the tube after removal from the anaerobic jar.

Milk.—Milk is an unsatisfactory medium for the growth of *B. tetani*, but

it may be improved by the addition of 1 per cent. of laked ox-blood. No obvious change has been observed in this medium after 3 weeks' incubation. No coagulation has been met with as claimed by Heller (1922) and Zeissler (1922–23).

Sugars.—The fermentative action of *B. tetani* upon sugars has been tested in peptone water containing 1 per cent. of the sugar and 1 per cent. of pepsin-digested blood. The reaction of the culture was tested with dye indicators. For the most part only glucose, lactose, maltose and saccharose were used. Durham's tubes were included to indicate gas production.

No fermentation of these sugars by 58 strains was observed during 4 weeks, although in three instances (TH 24, TS 4, TS 5) the pH of the glucose tube was reduced from 7·4 to about 6·5, acid to litmus. After heating and repeated subcultivation, two of these strains still tended to acidify glucose, but not constantly.

Coagulated serum.—The growth of *B. tetani* in coagulated serum was tested in 28 cases by adding 40 per cent. of horse-serum to ordinary broth and heating to 80° C. for one hour. Growth in this medium was feeble, but was improved by adding a few drops of laked ox-blood after coagulation. No very obvious effect was observed in these cultures, although after 2 to 3 weeks the clot was found to be split and to be more friable on shaking, and an occasional gas-bubble was seen. The coagulum was reduced in size and tended to be more transparent.

The proteolytic activity of B. tetani.—The slight but definite effect upon coagulated serum, and the more obvious though usually not intense action upon gelatine, suggests that *B. tetani* is endowed to a slight extent with proteolytic powers. This view is also supported by the frequent production of gas in most media. On the other hand, there is no evidence that this organism is capable of fermenting sugars.

CONCLUSIONS.

1. A method is described by which tetanus bacilli may be isolated easily from diverse materials.

2. The morphological and cultural characters of *B. tetani* are detailed from a study of 75 new strains isolated by this method.

Finally, I wish to express my thanks in a very special manner to Mr. J. E. Barnard, F.R.S., for taking all the photographs which illustrate this paper, and to Capt. S. R. Douglas, F.R.S., for selecting the specimens to be photographed. My thanks are also due to Dr. Alice Greaves for help in the examination of human fæces. Prof. J. McIntosh and Dr. S. L. Baker were good enough to send me specimens from two cases of human tetanus. The work was carried out under grants from the Medical Research Council.

The cultures upon which this study is based have been deposited in the National Collection of Type Cultures, Lister Institute, London. They are as follows:

From cases of human tetanus, 6 (TM 1–6).
From human fæces, 2 (TN 1 and 2).
From horse-dung, 34 (TH 1–34).
From soil, 33 (TS 1–34, excluding No. 12).

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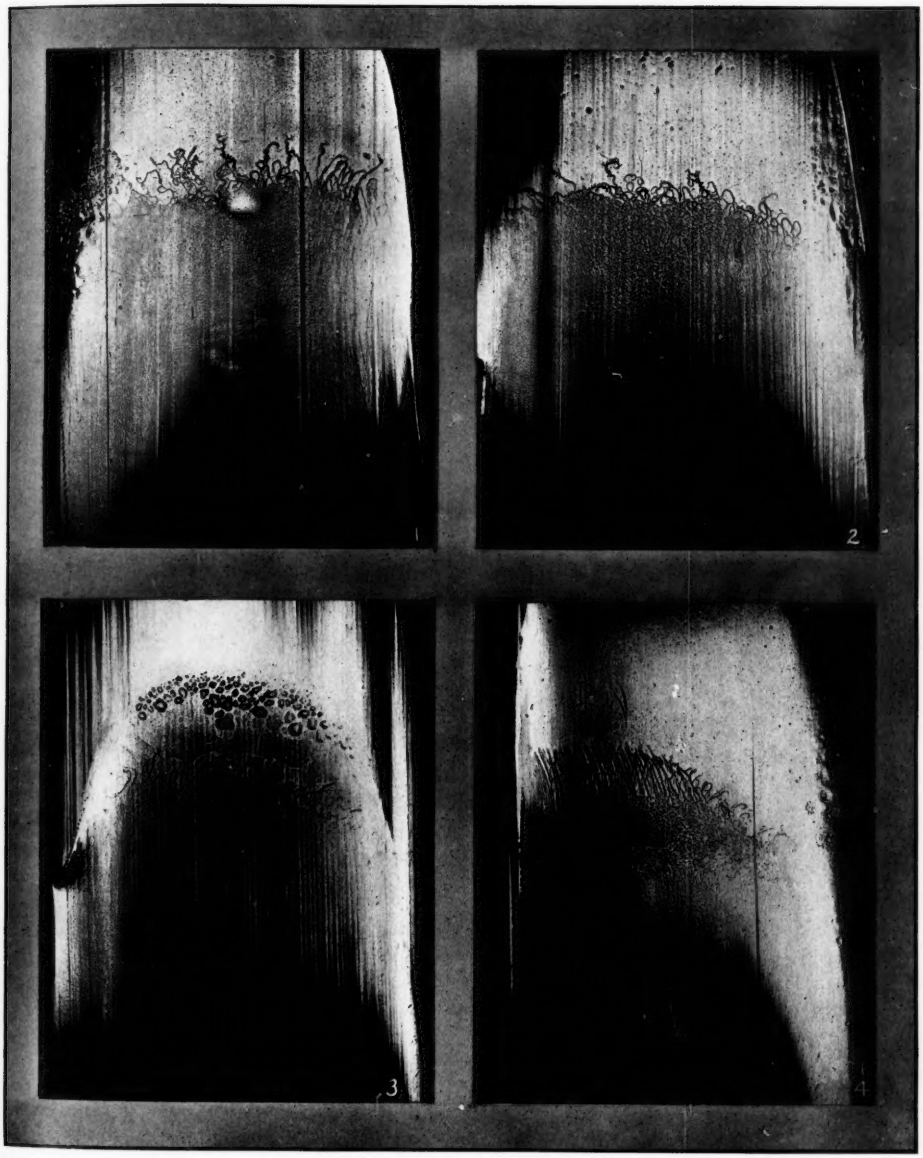
DESCRIPTION OF FIGURES.

Figs. 1–8.—Photographs × 5 of the top edge of 2-day slope cultures of tetanus bacilli. Figs. 1–4: Non-toxic, TH 12, TH 27, TS 13, TM 1. Figs. 5–8: Toxic, TH 29, TH 20, TN 1, TS 9. The sharp distinction between the length of the filaments of toxic and non-toxic strains is exaggerated by the photographs selected.

Figs. 9–26.—Photographs × 1280 of tetanus bacilli, all toxic except Figs. 10, 24, 25, 26. Fig. 9: TH 21, 17 hrs. Fig. 10: TS 10, 17 hrs. Fig. 11: TM 3, 2 day. Fig. 12: TM 5, 3 day, distal tip of protoplasm. Fig. 13: TH 29, do., do. Fig. 14: TH 22, 3 day, immature blue oval spores. Fig. 15: TH 21, 2 day, do. Fig. 16: Type strain I, 3 day, spores not quite spherical. Fig. 17: TH 21, 5 day, do. Fig. 18: TS 9, 3 day, do. Fig. 19: TH 21, 5 day, blue frame round spore. Fig. 20: TH 22, 3 day, oval spore. Fig. 21: TH 21, 6 day, do. Fig. 22: TH 20, 3 day, pyriform swelling. Fig. 23: TH 21, 6 day, fuchsin dots and pyriform swelling. Fig. 24: TM 1, 5 day, impure, fuchsin dots. Figs. 25, 26: Do., do., dumb-bells.

Figs. 27–29.—Non-agglutinating tetanus-like strains. Fig. 27: 140 N, 4 day. Fig. 28: 56 H, 2 day, blue spores. Fig. 29: 41 H, 4 day.

Fig. 30.—24-hour slope culture of soil containing tetanus bacilli. × 1. The fine filamentous growth of tetanus is seen at the top, and the limit of the contaminators half way up, the soil having been placed into the condensation water.



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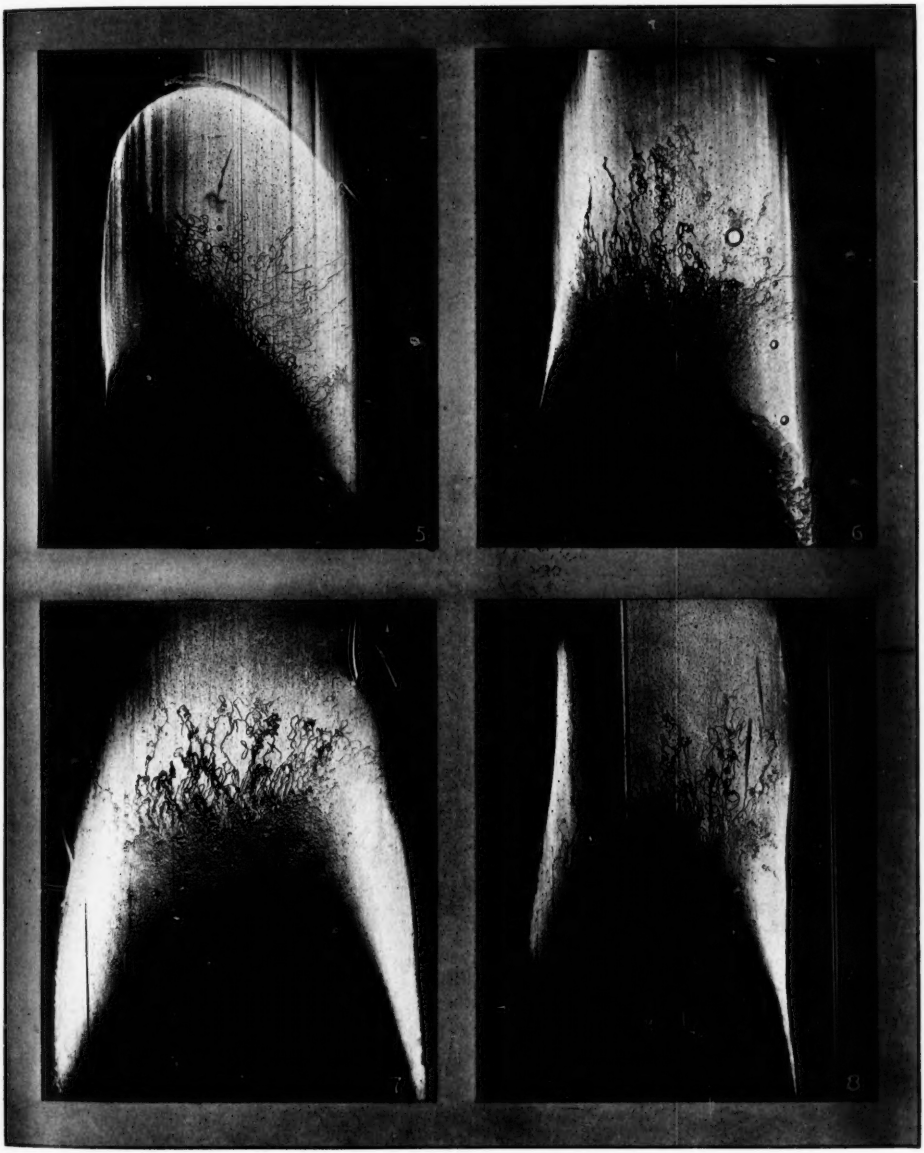

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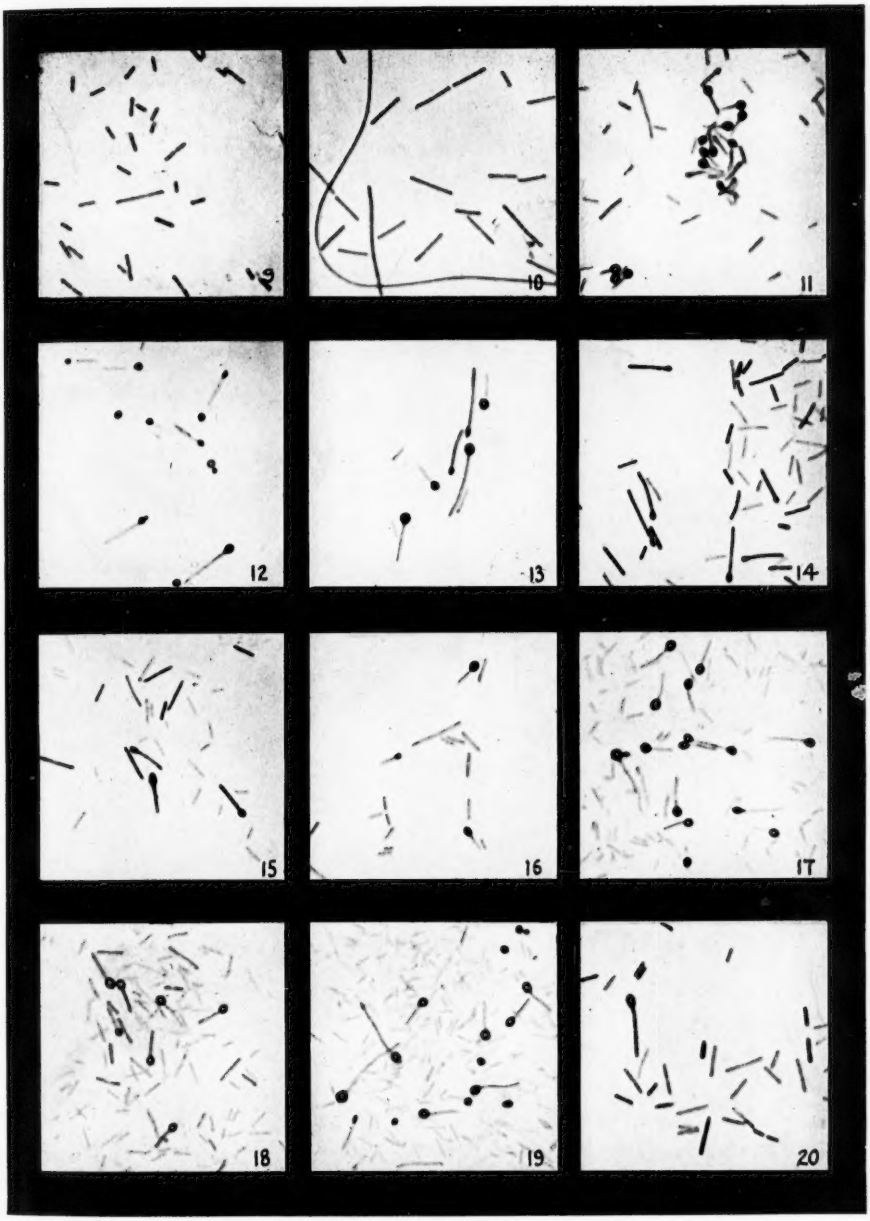

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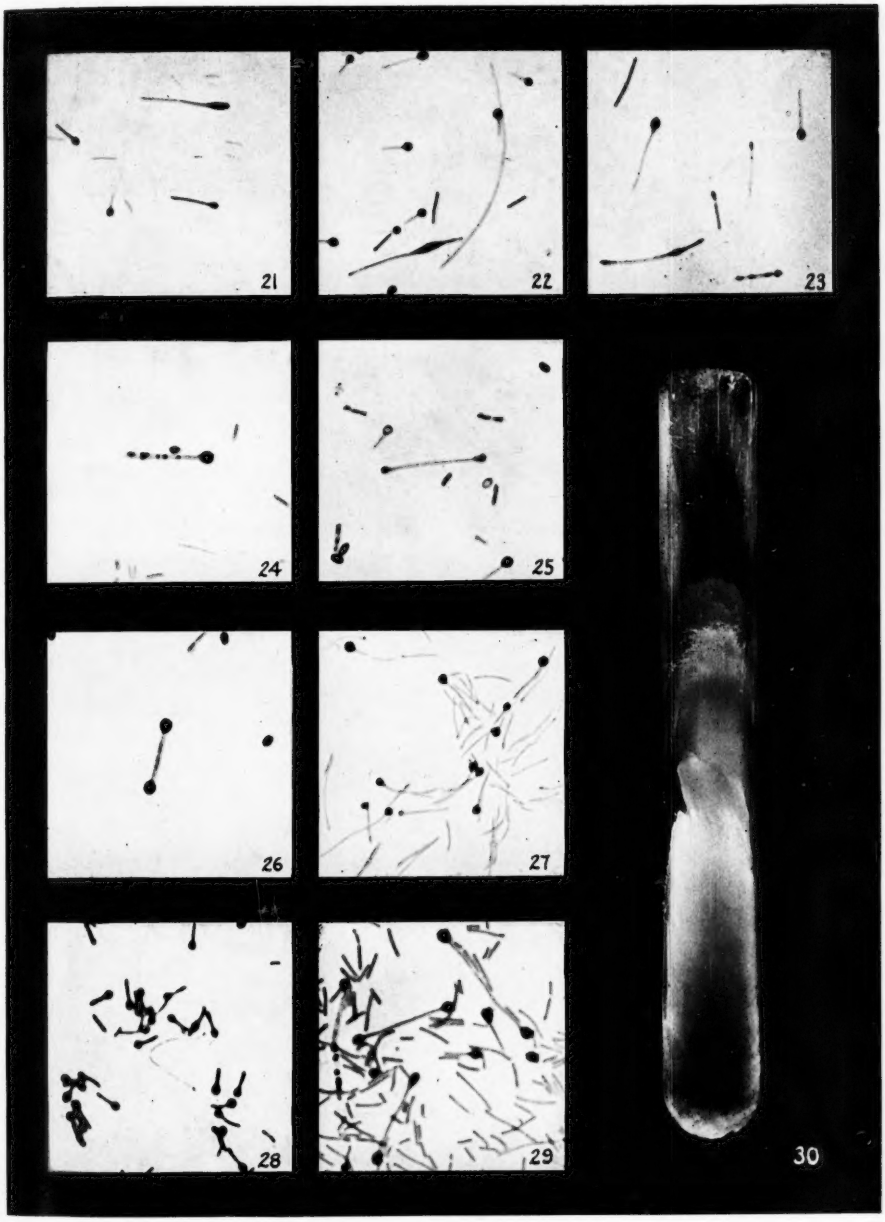

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INNERVATION AS A FACTOR IN THE EXPERIMENTAL PRODUCTION OF CANCER.

W. CRAMER.

From the Laboratories of the Imperial Cancer Research Fund, London.

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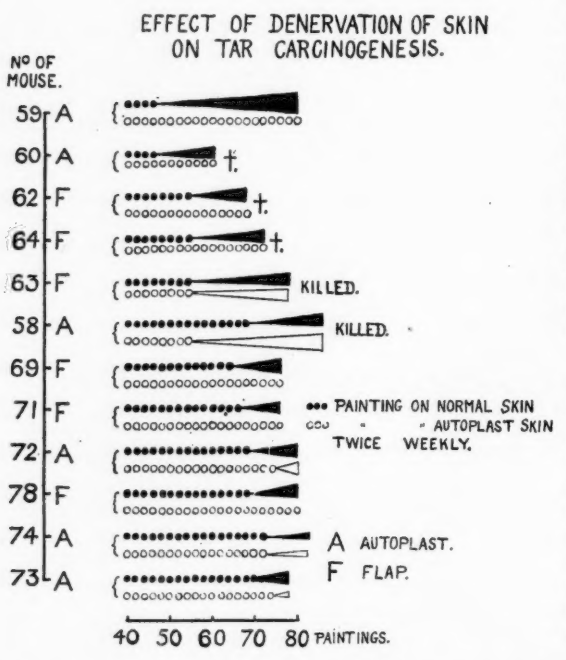
THE following observations were carried out to test whether the presence or absence of innervation in a piece of skin had any effect on the genesis of cancer. *A priori*, there are three different possibilities. If the presence of innervation is not an essential factor, then the application of tar to a denervated area of skin should produce cancer as readily there as on the normal skin. The second possibility is that the absence of innervation predisposes to cancer. If that were so, then the experimental production of cancer by tar-painting should be accelerated if the painting is applied to a denervated area of skin. From the premise that malignant new growths are devoid of nerves, it has been concluded that the absence of nerve control is, if not the cause, a material factor in the origin of cancer. It is obvious, however, that even if the premise is correct the conclusion does not necessarily follow. The third possibility is that innervation is an essential factor in the process of chronic irritation which leads to cancer. In that case denervation would delay or inhibit the experimental production of cancer by tar.

The experiments were carried out as follows: In a number of mice an oblong piece of skin on the middle of the back, about 2 cm. long and 1½ cm. broad, was completely severed from the surrounding skin and from the underlying tissues and sewn on again with fine catgut, so as to form an autoplast of skin, temporarily freed from nervous control. If the graft is unsuccessful the skin proliferates from the edges of the wound and spreads underneath the skin, which then dries up and falls off, so that instead of an autoplast of skin one has an area of regenerated skin at the site of the operation. The mice in which this process of skin regeneration occurred were rejected for the purpose of this experiment. Twenty mice were prepared in this way with a successful skin-autoplast. In a second series of 23 mice an elongated flap of skin was cut, which remained connected anteriorly in the middle line with the skin of the back by a narrow bridge of tissue 3 to 4 millimetres in breadth. After lifting up this flap and severing it completely from the underlying tissue it was stitched up again with catgut. This procedure closely resembled that of grafting in producing a denervated area. When after about two weeks healing was complete, tar-painting was begun twice weekly in the centre of the skin-autoplast or skin-flap, and, as a control, also on the normal skin on the nape of the neck. Altogether 43 mice were subjected to this treatment. At the end of 9 months 12 of the surviving mice had developed tumours and 5 had remained completely negative. All the 12 mice had a tumour on the

normal skin. Only 5 mice had developed a tumour also on the skin-flap or autoplast. Of these only one mouse (No. 58) developed a tumour on the skin-autoplast previous to showing a tumour on the normal skin, while 3 appeared very late on the skin-autoplast, requiring more than 70 paintings twice weekly.

The result can be seen at a glance in the accompanying chart. It is interesting to note that the tumour which arose early in the skin-autoplast of mouse No. 58 was much less malignant in character and more limited in its spread than the tumour which appeared 7 weeks later on the normal skin of the same mouse (see Figs. 1 and 2). No such difference was found, however, in the two tumours which appeared in mouse No. 63; they showed approximately equal degrees of malignancy.



In interpreting the results it must be taken into consideration that presumably nerve regeneration took place in the skin-autoplast, so that the autoplast remained completely free from nervous control only during the first two or three months after the operation, and that then nervous control was eventually re-established. The regeneration of a peripheral nervous plexus has been studied by Meeks (1911) in the intestine of cats and dogs. He has shown that after transection the regeneration of Auerbach's plexus is not completed until at least four months have elapsed. In the mouse this process, like all processes of healing and repair, is probably more rapid. One must assume therefore that after two to three months the difference in innervation between the normal skin and the skin-autoplasts or skin-flaps gradually disappears, and with it the difference in the reaction to chronic irritation between the normal skin and the originally denervated area. This would account for the three tumours which appeared in the mice Nos. 72, 73 and 74 (see chart) after more than eight months of paintings.

The possibility that different areas of the skin of the back differ in their susceptibility to tar-painting sufficiently to account for the differences observed in this experiment can be ruled out by a series of observations of Dr. J. A.

FIG. 1.—Mouse 58. Ether-tar tumour on skin autoplast.

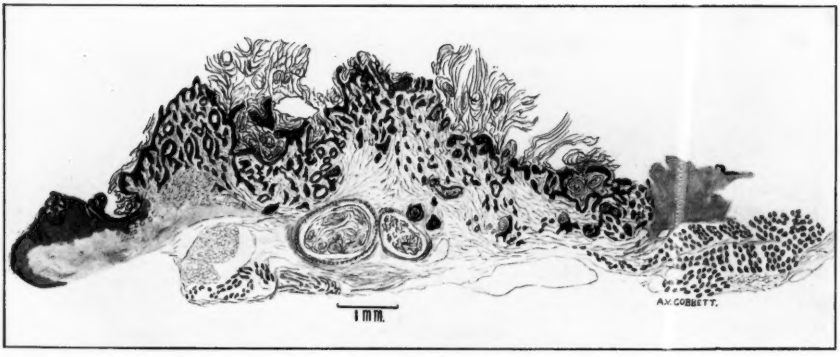


FIG. 2.—Mouse 58. Ether-tar tumour on normal skin.

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Murray and another series carried out by me (unpublished observations). Dr. Murray has also found (unpublished observations) that when two different sites of normal skin are tarred simultaneously with the same tar preparation, the appearance of a tumour on one of the sites does not inhibit the appearance of another tumour on the second site.

The results of the present experiment, therefore, show quite clearly that lack or impairment of nervous control in an area of skin does not cause cancer, and does not even predispose to it. There is on the contrary clear indication that in the process of chronic irritation, which leads eventually to the development of cancer, the presence of an efficient nervous control is an important factor.

It is not as yet possible to define precisely the part played by the nervous control in the process of chronic irritation which leads to cancer. But it may be recalled that the response to an acute irritant such as mustard oil, which produces active vasodilation, and thereby the condition of inflammation, has been shown by Bruce (1913) to be dependent on the integrity of a peripheral axon reflex. The vasodilation and the inflammatory reaction fail to appear after the application of the acute irritant if the peripheral nerves involved in the axon reflex are degenerated. Similarly, Krogh (1920) has demonstrated more recently that the response of the capillaries to local stimulation is due to such local axon reflexes.

These experimental observations are in agreement with the clinical observations of Lenthal Cheatle (1903, 1908) on the relationship of cancer and nerve areas, in which he has shown that there is frequently a distinct relationship between the definite nerve areas and the incidence and spread of rodent ulcer, and sometimes even of carcinoma. Particularly striking are the cases recorded by him, where an extensively spreading rodent ulcer was sharply limited in its spread by an area of skin which had become anæsthetic and analgesic. Of special interest in connection with the work on experimental tar cancer are his observations on the incidence of tar cancer in man, where the incidence frequently coincides with Head's " maximum points."

Cheatle points out that " the maximum point of an area does not claim to possess qualities and properties which do not exist otherwise on that area, but only to possess them in a more accentuated degree." He refers to a case of tar cancer in the forearm of a man whose whole arm was exposed to and suffered from the effects of tar irritation, but the place at which the squamous epithelium became cancerous was on the maximum point of the first dorsal root. He suggests that if the area of irritation includes a maximum point, the most likely place for the incidence of cancer will be that maximum point. If, on the other hand, the maximum point is not included in the area exposed to chronic irritation, then " the irritation ought to be greater or of longer duration to produce the same effect."

These considerations may possibly explain those rare cases where the single application of an irritant such as tar has, even in man, produced a carcinoma in the course of a few weeks.

CONCLUSION.

Loss of nervous control in an area of skin does not predispose to cancer. On the contrary the presence of a peripheral nervous mechanism is an essential

factor in the process of chronic irritation which leads to the development of cancer.

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THE SEXUAL GLANDS AND METABOLISM.—II. INFLUENCE OF EMULSIONS OF TESTIS AND PROSTATE UPON THE NITROGEN METABOLISM OF RABBITS.

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From the Lister Institute, London.

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IN this paper, the aim of which is to try to elucidate the function of the prostate, the literature referring to this gland only will be discussed. The next paper, dealing with many other experiments on dogs and rabbits injected with an emulsion of sexual glands, testis and ovaries, will contain a review of the literature concerning these latter glands. Up to the present time the function of the prostate and particularly its significance as an endocrine gland remain almost unknown.

The prostate is physiologically related to the *testis*, and the integrity of the latter is necessary for the full development of the former. After castration of man or animal, the prostate atrophies to a marked degree (see references in Wallace, 1907, and Biedl, 1916). Atrophy of this gland was also found in cases of gonad deficiency (Morris, 1916; Lisser, 1923). Walker (1900), by injecting a glycerine testicular extract into dogs, did not prevent the atrophy of the prostate after castration.

Zindel (1913) several times exposed the testes of dogs to Roentgen rays. On post-mortem examination typical changes were present in the testes, but the prostate was normal in every respect. However, Nemenov (1922) found an hypertrophy of the prostate of dogs after the exposure of their testes to Roentgen rays. Histologically, atrophy of the semeniferous cells in the testis was revealed and an increase in the number of Leydig's cells. Therefore Nemenov concludes that the hypersecretion of the testis produced hypertrophy of the prostate.

The development of the prostate is also influenced by the *pituitary gland*:

atrophy of the prostate was found in experimental hypopituitarism (Biedl, 1916), and in patients with signs of decreased function of the pituitary (Biedl, 1916; Lisser, 1923). Goetsch (1916), on the other hand, found that feeding rats with the anterior part of the hypophysis produced more marked growth and earlier development of the genital organs, prostate included, than in control animals.

The only available data concerning the possible *inner secretion of the prostate* should be mentioned here. It is difficult to draw any definite conclusions concerning the inner secretion of prostate from observations on human patients suffering from hypertrophy of this gland, because in this pathological condition not only prostatic tissue is involved, but the submucous urethral glands as well (Walker, 1922). Serralach and Pares (1907) removed the prostate from dogs, after which atrophy of the testes resulted with absence of spermatogenesis and ejaculation. This was partly prevented by the ingestion of glycerine extracts of prostate. The above-mentioned results of prostatectomy disappeared after 7 to 12 months owing to the regeneration of the prostatic tissue, the last remnants of which it is very difficult to remove in a dog. These authors also found that the ingestion by patients of a testicular extract produced hypersecretion of prostate. Biedl (1916) was unable to find any changes in the sexual glands of one successfully prostatectomized dog one year after the operation. Macht and Matsumoto (1920) and Macht (1921) found that the effect of feeding prostate gland to tadpoles was to hasten the metamorphosis of frog larvæ and salamander larvæ, but unlike the thyroid gland, the prostate did not produce shrinkage of the animals; it indeed had a tendency to produce an increase in their size and weight. It was also found that the prostate bears no relation to the contractions and tonicity of various genito-urinary organs and to the intelligent behaviour or "mental efficiency" of albino rats. Rogoff and Rosenberg (1922) were able to increase growth or hasten slightly the metamorphosis of tadpoles by feeding them with a number of substances, including prostate, but none was so efficient as thyroid.

Experiments to discover the influence of different extracts of prostate when injected intravenously have not given any reliable results because of their non-specific actions, commonly following the injections of all tissue extracts. This was not always realized by the workers (Thaon, Posner and Kohn and others).

In 1920 one of us (Korenchevsky and Bogoslovsky) performed 6 metabolism experiments on 2 normal and 2 castrated dogs (of large size). Daily, during the period of injections, 10 c.c. of a fresh aseptic emulsion of half a prostate or quarter of a testis of a dog were given subcutaneously to each experimental animal. The first control period, before the injections, lasted 3 days, the period of injections 2 days, followed by the second control period, which usually lasted only 1 day. During the control periods 10 c.c. of saline solution were injected daily into each animal. The technique of the metabolism experiments was described elsewhere (1925). The chief results obtained are summarized in Table No. 1, and are as follows:

Injections of prostate emulsion did not produce any harmful effect upon the animals. No constant or definite effect upon the nitrogen metabolism was found in 2 normal dogs, Nos. 3 and 4.

TABLE I.—*The Percentage Change from the Normal in the Nitrogen and Gaseous Metabolism of Four Dogs after Injections of Emulsions of Prostate and of Testis and Prostate.*

No. of dog.	Weight.		Diuresis.		N. in urine.		Gaseous metabolism. H_2O.		CO_2.		O_2.		Remarks.
	Inj. p.	Cont. p.	Inj. p.	Cont. p.	Inj. p.	Cont. p.	Inj. p.	Cont. p.	Inj. p.	Cont. p.	Inj. p.	Cont. p.	
Normal dogs:													
3.	+0·3	+0·7	+5·9	+8·0	+1·2	−15·6	−16·4	−15·1	−4·5	−8·9	+4·3	+19·0	Prostate emulsion.
4.	+0·15	+2·03	−12·5	−6·5	−0·1	−1·4	−35·9	−43·5	−20·8	−21·4	−14·1	−21·2	,, ,,
Average	+0·09	+1·36	−3·3	+0·8	+0·6	−8·5	−26·2	−29·3	−12·7	−15·2	−4·9	−1·1	
Castrated:													
2.	+0·5	+0·5	+4·3	+3·1	+0·9	+4·2	−9·6	−15·5	−8·7	−9·3	−6·9	−19·2	Prostate emulsion.
	+0·2	+0·3	+62·2	+27·0	+9·1	+7·8	−1·9	−2·6	+1·8	+2·2	+3·8	−7·9	,, ,,
5.	+0·2	+0·08	+13·0	+10·0	+18·2	+11·4	+11·9	−2·9	+4·0	+10·1	+28·5	+10·9	,, ,,
Average	+0·3	+0·3	+26·5	+13·4	+9·4	+7·8	+0·1	−7·0	−1·0	+1·0	+8·5	−5·4	
2.	+1·1	+1·8	+4·0	+23·0	+17·4	−20·6	−7·5	−5·9	+1·6	+1·2	+0·5	+3·7	Testis and prostate emulsion.

TABLE II.—*The Influence of Subcutaneous Injections of Emulsions of Kidney and of Both Testis and Prostate upon the Nitrogen Metabolism of Rabbit No. 8 (castrated).*

No. of period.	Weight of rabbit at end of each period in gm.	Total intake of N. in gm.	Excretion of urine in c.c.	N. in urine in gm.	Weight of dried fæces in gm.*	N. in fæces in gm.*	Total output of N. in gm.	Balance of N. in gm.	Remarks.
J.	2430	7·623	455·0	6·499	92·1	1·084	7·583	+0·040	I control period.
K.	2457	7·652	429·3	6·175	92·1	1·084	7·259	+0·393	Inj. of emulsion of *kidney*.
L.	2460	7·669	487·7	6·367	92·1	1·084	7·451	+0·218	II control period.
M.	2460	7·651	466·1	6·322	92·1	1·084	7·406	+0·245	III control period.
O.	2350	8·227	504·1	9·074	98·1	1·478	10·552	−2·325	I control period.
P.	2295	8·230	474·0	8·800	98·1	1·478	10·278	−2·048	Inj. of emulsion of *prostate and testis*.
R.	2248	8·217	479·6	10·11	98·1	1·478	11·588	−3·371	II control period.
Q.	2195	8·217	445·6	9·530	98·1	1·478	11·008	−2·791	III control period.
S.	2152	8·217	612·3	9·493	98·1	1·478	10·971	−2·754	IV control period.

* NOTE.—Owing to the ball-like formation of rabbits' fæces and to the varying degree of activity of peristalsis, it is impossible to separate accurately the fæces belonging to each experimental period. Therefore the fæces were collected during the whole experiment and N. content was divided according to the length of time of each experimental period (see Tables II–VII).

When the same emulsion was injected into the *castrated* dogs Nos. 2 and 5, an increase was produced in the diuresis and in the nitrogen metabolism both in the period of injection and the following control period. But when an emulsion of both prostate and testis were injected simultaneously into dog No. 2, the rise in the nitrogen metabolism was much more pronounced, suggesting the possible synergic interrelation of these glands as regards the nitrogen metabolism. The data concerning gaseous metabolism were contradictory in the different experiments. From these few preliminary experiments an hypothesis was suggested that the prostate has a function of inner secretion, by means of which nitrogen metabolism may be increased.

THE PRESENT INVESTIGATION: TECHNIQUE.

Sixteen experiments were performed on 6 male rabbits, 2 of which, Nos. 8 and 14, were castrated. Each experiment was divided into 4 periods, namely, the first control period of 3 days before the injections, the period of injections lasting 3 days, followed by 2 or 3 control periods, each of which usually lasted 3 or sometimes one of them only 2 days.

The technique used for the study of the nitrogen metabolism of rabbits was described in a paper published elsewhere (Korenchevsky, 1925), but it is necessary to mention a few other technical details here. After the rabbits had been chosen for the experiments they were fed on a special minimal ration, which was just sufficient to keep their weight constant, and which was given in the same amount daily during the rest of their lives. They ate their ration eagerly, and usually finished it 2 to 3 hours after it had been given to them. The amount of oats given to each rabbit varied from 40–70 gm., and cabbage from 100–120 gm.

A large amount of well-mixed oats was stored for the duration of the experiment. Cabbage was also stored in stoppered jars in the cold room, having been chopped up and mixed. The rabbits were catheterized at the beginning and end of each experimental period, and their bladders washed two or three times with warm saline solution.

During all the control periods, 1 c.c. of saline solution was injected subcutaneously daily into each rabbit and during the injection period 1 c.c. of emulsion. The latter was prepared in the following manner: On the day of injection a rabbit was killed, and the testis, prostate and kidney were immediately cut out with sterilized implements. An emulsion was prepared from the whole dorsal part of the prostate, the weight of which roughly corresponded to half that of the testis or $\frac{1}{5}$ of the kidney. Therefore these amounts of testis and of kidney were usually taken for the emulsions. The glands, after removal, were chopped with scissors, thoroughly ground in a mortar emulsified with 10 c.c. of saline solution and filtered through gauze. All the manipulations were performed aseptically. The resulting emulsion was opaque, did not contain any gross particles, and contained about 0·23 per cent. nitrogen. Therefore each injection of 1 c.c. into a rabbit contained about 2·3 mg. nitrogen. In order to observe the combined influence of prostate and testis, the emulsions of both were mixed together in equal parts. So 1 c.c. of testis emulsion corresponded to $\frac{1}{10}$ to $\frac{1}{20}$ testis, and 1 c.c. of prostate emulsion to

TABLE III.—*The Influence of Subcutaneous Injections of Emulsions of Prostate, Testis, and both Testis and Prostate upon the Nitrogen Metabolism of Rabbit No. 14 (castrated).*

No. of period.	Weight of rabbit at end of each period in gm.	Total intake of N. in gm.	Excretion of urine in c.c.	N. in urine in gm.	Weight of dried fæces in gm.	N. in fæces in gm.	Total output of N. in gm.	Balance of N. in gm.	Remarks.
J.	3195	9·820	612·4	7·893	144·5	2·113	10·006	—0·186	I control period.
K.	3230	9·795	487·7	8·015	144·5	2·113	10·128	—0·333	Inj. of emulsion of *prostate.*
L.	3185	9·812	583·3	8·600	144·5	2·113	10·713	—0·901	II control period.
M.	3170	9·802	549·6	8·128	144·5	2·113	10·241	—0·439	III control period.
O.	3260	10·479	529·5	8·716	137·1	2·181	10·897	—0·418	I control period.
P.	3252	10·468	488·0	8·110	137·1	2·181	10·291	+0·177	Inj. of emulsion of *testis.*
R.	3235	10·471	441·1	8·053	137·1	2·181	10·234	+0·237	II control period.
Q.	3230	10·471	471·4	8·073	137·1	2·181	10·254	+0·217	III control period.
S.	3222	10·471	430·5	8·090	137·1	2·181	10·271	+0·200	IV control period.
T.	3160	9·793	518·1	8·431	127·2	1·703	10·134	—0·341	I control period.
U.	3175	9·793	480·8	8·500	127·2	1·703	10·203	—0·410	Inj. of emulsion of *testis and prostate.*
V.	3155	9·793	514·5	9·232	127·2	1·703	10·935	—1·142	II control period.
W.	3125	9·793	483·1	8·994	127·2	1·703	10·697	—0·904	III control period.

TABLE IV.—*The Influence of Subcutaneous Injections of Emulsions of Prostate, Kidney, and both Testis and Prostate upon the Nitrogen Metabolism of Rabbit No. 16 (non-castrated).*

No. of period.	Weight of rabbit at end of each period in gm.	Total intake of N. in gm.	Excretion of urine in c.c.	N. in urine in gm.	Weight of dried fæces in gm.	N. in fæces in gm.	Total output of N. in gm.	Balance of N. in gm.	Remarks.
J.	3000	9·804	633·6	8·734	114·9	1·103	9·837	—0·033	I control period.
K.	3015	9·793	599·0	9·114	114·9	1·103	10·217	—0·424	Inj. of emulsion of *prostate.*
L.	2995	9·800	608·1	9·620	114·9	1·103	10·723	—0·923	II control period.
M.	3000	9·793	525·2	8·756	114·9	1·103	9·859	—0·066	III control period.
O.	3035	10·460	588·0	9·490	119·6	1·137	10·627	—0·167	I control period.
P.	3010	10·481	632·4	9·798	119·6	1·137	10·935	—0·454	Inj. of emulsion of *testis and prostate.*
R.	2995	10·471	518·1	10·49	119·6	1·137	11·627	—1·156	II control period.
Q.	2970	10·471	555·4	9·606	119·6	1·137	10·743	—0·272	III control period.
S.	2975	10·471	514·5	9·212	119·6	1·137	10·340	+0·121	IV control period.
T.	2962	9·793	529·6	9·270	126·2	1·352	10·622	—0·829	I control period.
U.	2965	9·793	462·1	8·992	126·2	1·352	10·344	—0·551	Inj. of emulsion of *kidney.*
V.	2970	9·793	507·6	8·577	126·2	1·352	9·929	—0·136	II control period.
W.	2970	9·793	520·4	8·710	126·2	1·352	10·062	—0·269	III control period.

TABLE V.—*The Influence of Subcutaneous Injections of Emulsions of Prostate and of Testis upon the Nitrogen Metabolism of Rabbit No. 17 (non-castrated).*

No. of period.	Weight of rabbit at end of each period in gm.	Total intake of N. in gm.	Volume of urine excreted in c.c.	N. in urine in gm.	Weight of dried fæces in gm.	N. in fæces in gm.	Total output of N. in gm.	Balance of N. in gm.	Remarks.
J.	2655	8·697	492·2	6·737	99·9	1·313	8·050	+0·647	I control period.
K.	2675	8·719	472·3	7·046	99·9	1·313	8·359	+0·360	Inj. of emulsion of *prostate.*
L.	2655	8·715	525·1	7·460	99·9	1·313	8·773	—0·058	II control period.
N.	2660	8·717	441·1	7·180	99·9	1·313	8·493	+0·224	IV* control period.
T.	2510	8·715	443·4	7·827	95·61	1·300	9·127	—0·412	I control period.
U.	2500	8·715	448·0	7·587	95·61	1·300	8·887	—0·172	Inj. of emulsion of *testis.*
V.	2495	8·715	423·6	7·813	95·61	1·300	9·113	—0·398	II control period.
W.	2485	8·715	464·4	7·595	95·61	1·300	8·895	—0·180	III control period.

*Nitrogen in the rabbit used in No. 18 (Table VI) urinary analyses was lost in the III control period.

$\frac{1}{10}$ of the dorsal lobe of the prostate. 1 c.c. of the combined emulsion contained $\frac{1}{40}$ testis and $\frac{1}{20}$ prostate, and 1 c.c. of kidney emulsion $\frac{1}{50}$ of kidney. The injections of these emulsions produced only a slight increase up to 0·5°C. in the temperature of the rabbits, and no difference at all was noticed between the rise of temperature caused by the different emulsions. This increase in the temperature did not produce any noticeable increase in the nitrogen metabolism, as was indicated by the effect of injections of the emulsions of testis or kidney. In order to avoid any accidental mistakes, experiments were performed on 6 animals at a time, some rabbits being given injections of one emulsion and others of other emulsions. Kidney emulsion was made for control experiments in order to see whether the influence of injections of testis and prostate upon metabolism was specific.

DESCRIPTION OF THE EXPERIMENTS.

The actual figures of nitrogen intake and output for each period were calculated per week so as to be comparable with one another, and the results of the experiments are summarized in Tables, Nos. II–VII. In Table VIII are summarized results which show the percentage change in the nitrogen excretion in the urine during the period of injection and the two or three following control periods, compared with that of the same animals during the first control period before the injections.

From these tables it is clear that in all experiments injections of kidney, testis and prostate each showed a *constant* effect upon the nitrogen metabolism. Not only kidney, but testis also produced a decrease in the nitrogen metabolism seen clearly in the injection period, and usually in the two succeeding control periods as well. There was practically no difference between the influences of testis and kidney. Therefore this effect of testis upon the nitrogen metabolism seems not to be specific. Probably it must be attributed to some substances contained in other organs as well. We consider this influence of testis emulsion striking, because castration in rabbits produces a fall of nitrogen metabolism as well (Korenchevsky, 1925). We are inclined to explain these contradictory results by the possible presence in the testis of the rabbit of two different principles, one increasing, the other decreasing nitrogen metabolism, the latter influence prevailing when testis is injected as an emulsion. Another possible explanation may be that injections of emulsions of testis or of kidney stimulate those endocrine glands which decrease nitrogen metabolism—for example, pancreas—or they depress those endocrine glands which increase nitrogen metabolism—for example, thyroid.

No difference was found between the reactions of castrated (Nos. 8 and 14) or of non-castrated animals (Nos. 16, 17, 18 and 19).

Injections of emulsion of prostate or of prostate and testis simultaneously increased the excretion of nitrogen in urine on an average by 9 per cent. (maximum 11·4 per cent., see Table VIII). It is noteworthy that this effect was always most pronounced and sometimes was only present in the control period following the period of injections.

Diuresis decreased in most cases, but in a very varying degree, sometimes the change being within the normal fluctuations.

TABLE VI.—*The Influence of Subcutaneous Injections of Emulsions of Prostate, Testis, and both Testis and Prostate upon the Nitrogen Metabolism of Rabbit No. 18 (non-castrated).*

No. of period.	Weight of rabbit at end of each period in gm.	Total intake of N. in gm.	Volume of urine excreted in c.c.	N. in urine in gm.	Weight of dried fæces in gm.	N. in fæces in gm.	Total output of N. in gm.	Balance of N. in gm.	Remarks.
J.	2800	8·725	567·0	7·753	104·1	1·070	8·823	−0·098	I control period.
K.	2770	8·721	620·0	7·901	104·1	1·070	8·971	−0·250	Inj. of emulsion of *prostate*.
L.	2765	8·722	576·4	8·225	104·1	1·070	9·295	−0·573	II control period.
N.	2760	8·720	574·0	7·727	104·1	1·070	8·797	−0·077	IV control period.
O.	2775	9·381	611·3	8·563	89·22	1·133	9·696	−0·315	I control period.
P.	2730	9·418	556·9	8·541	89·22	1·133	9·674	−0·256	Inj. of emulsion of *testis and prostate*.
R.	2735	9·393	451·5	9·438	89·22	1·133	10·571	−1·178	II control period.
Q.	2720	9·393	478·4	8·564	89·22	1·133	9·697	−0·304	III control period.
S.	2715	9·393	476·0	8·401	89·22	1·133	9·534	−0·141	IV control period.
T.	2645	8·715	557·6	8·385	93·84	1·098	9·483	−0·768	I control period.
U.	2600	8·715	490·0	8·037	93·84	1·098	9·135	−0·420	Inj. of emulsion of *testis*.
V.	2610	8·715	392·0	8·165	93·84	1·098	9·263	−0·548	II control period.
W.	2590	8·715	471·4	8·540	93·84	1·098	9·638	−0·923	III control period.

TABLE VII.—*The Influence of Subcutaneous Injections of Emulsions of Kidney, Testis, and both Testis and Prostate upon the Nitrogen Metabolism of Rabbit No. 19 (non-castrated).*

No. of period.	Weight of rabbit at end of each period in gm.	Total intake of N. in gm.	Volume of urine excreted in c.c.	N. in urine in gm.	Weight of dried fæces in gm.	N. in fæces in gm.	Total output of N. in gm.	Balance of N. in gm.	Remarks.
J.	3036	11·954	504·1	9·352	160·7	1·968	11·320	+0·634	I control period.
K.	3015	11·954	522·6	8·866	160·7	1·968	10·834	+1·120	Inj. of emulsion of *kidney*.
L.	3075	11·959	423·0	8·864	160·7	1·968	10·832	+1·127	II control period.
M.	3050	11·945	577·5	8·810	160·7	1·968	10·778	+1·167	III control period.
O.	3095	12·638	571·6	10·67	144·0	1·549	12·219	+0·419	I control period.
P.	3022	12·631	584.9	10·81	144·0	1·549	12·359	+0·272	Inj. of emulsion of *testis and prostate*.
R.	3010	12·627	458·5	11·16	144·0	1·549	12·709	−0·082	II control period.
Q.	2985	12·627	452·7	11·06	144·0	1·549	12·609	+0·018	III control period.
S.	2984	12·627	371·0	10·62	144·0	1·549	12·169	+0·458	IV control period.
T.	2890	11·949	459·7	10·00	161·1	2·310	12·310	−0·361	I control period.
U.	2895	11·949	385·0	9·598	161·1	2·310	11·908	−0·041	Inj. of emulsion of *testis*.
V.	2905	11·949	402·6	10·03	161·1	2·310	12·340	−0·391	II control period.
W.	2900	11·949	382·6	9·922	161·1	2·310	12·232	−0·283	III control period.

If the figures of nitrogen metabolism during the first control periods of each experiment on the same rabbit are compared with one another, certainly some idea of the conditions of metabolism during the intervals of 3–4½ weeks between the experiments could be obtained. During these intervals animals were given a rest and were still fed on the chosen ration. In all cases the nitrogen metabolism of the rabbits became increased, although in a varying degree, towards the end of the observation, *i.e.* in the third experiment. For example, during the three consecutive first control periods of rabbit 19 (Table VII, periods J., O. and T.) the total output of nitrogen was about 11·3 gm., 12·2 gm. and 12·3 gm. respectively. It is true that during the second experiment on each rabbit the nitrogen intake was larger because the nitrogen content of the cabbage used in this series was higher. However, this does not explain the increased nitrogen output, because this increase was larger than that of the nitrogen intake. At the beginning of the third series of experiments, when the nitrogen was identical with that of the first experiment, there was still an increase in the nitrogen metabolism.

This change cannot be attributed to the variations in the nitrogen excreted in the fæces, because this remained almost constant in all three experiments on rabbits 14, 16 and 17. Although fluctuations in the nitrogen excreted by the fæces of some rabbits influenced the total nitrogen output and the nitrogen balance to some extent, they did not account for the gradual increase in the nitrogen balance as the rabbit grew older. The causes of this phenomenon will be explained more definitely when our experiments to elucidate this subject are finished.

In our experiments we obtained an increase in the nitrogen metabolism not exceeding 11 per cent. after injections of prostate. Taking into consideration the accuracy of our technique and the invariability of the results obtained from all rabbits experimented upon, we feel justified in considering this comparatively small increase in the nitrogen metabolism as being beyond experimental error, and quite significant as a change in the metabolism. This conclusion is also corroborated by the somewhat similar results obtained by one of us on dogs. With regard to the small changes produced by these injections, it is necessary to take the following facts into consideration: The glands of internal secretion could be divided into two groups. Removal of those belonging to one group produces drastic effects and sooner or later becomes fatal. The hormones from such glands, when administered, usually have a very strong and rapid effect, *e. g.* thyroid. The removal of those glands in the second group is followed by a much smaller effect, which develops slowly, and the life of the animal or man without them does not seem to be shortened. Accordingly the action of the hormones of the latter glands would be expected to be feeble and slow. The sexual glands, prostate included, evidently belong to the second group, and a rise in the metabolism of 11 per cent. caused by one of them seems quite significant. One point more should be mentioned. An emulsion of a gland is certainly not an ideal form in which to study the influence of the hormones of this gland. It always may contain many principles acting in opposite directions. For example, in a crude insulin fraction two substances are present, one increasing, the other decreasing the blood-sugar (Ashby, 1923).

TABLE VIII.—*Percentage Change from the Normal in the N. Output in the Urine of Rabbits after Injections of Different Emulsions.*

No. of rabbit	Inj. p.	II cont. p.	III cont. p.	IV cont. p.	Gland injection.
8.	−5·0	−2·0	−2·7	—	Kidney emulsion.
16.	−2·9	−7·5	−6·0	—	
19.	−5·2	−5·2	−5·8	—	
Average	−4·4	−4·9	−4·8	—	
14.	−7·0	−7·6	−7·4	−7·2	Testis emulsion.
17.	−3·1	−0·2	−3·0	—	
18.	−4·2	−2·6	+1·8	—	
19.	−4·0	+0·3	−0·8	—	
Average	−4·6	−2·5	−2·4	−7·2	
14.	+1·5	+9·0	+2·9	—	Prostate emulsion.
16.	+4·4	+10·2	0	—	
17.	+4·6	+10·7	—	+6·6	
18.	+1·9	+6·0	—	0	
Average	+3·1	+9·0	+1·5	+3·3	
8.	−3·0	+11·4	+5·0	+4·6	Prostate and testis emulsion.
14.	+0·8	+9·5	+6·7	—	
16.	+3·2	+10·5	+1·2	+2·9	
18.	0	+10·2	0	−1·9	
19.	+1·3	+4·6	+3·7	−0·5	
Average	+0·5	+9·2	+3·3	+1·3	

Accurate and undeniable conclusions concerning the inner secretion of a gland can only be obtained when its active principle has been isolated in a more or less pure form. This certainly also applies to the prostate, the inner secretion of which we have suggested.

SUMMARY.

Sixteen nitrogen metabolism experiments were performed on six rabbits, into which freshly-prepared homologous emulsions of kidney, testis and prostate or both testis and prostate were injected.

Testis emulsion, similarly to that of kidney, when injected subcutaneously into rabbits slightly decreased the nitrogen metabolism during the period of injections, and in most cases during the succeeding 6–9 days. This effect of testis emulsion cannot be considered a specific one, because of the similar results obtained with kidney emulsion.

An emulsion of prostate, when alone or in combination with testis, when injected in the amounts given in the above experiments, increased the nitrogen metabolism up to 11 per cent., particularly during the two or three days following the period of injections.

No difference was found between the reactions of castrated or of non-castrated rabbits.

Taking into consideration that experiments with prostate emulsion previously made by one of us on dogs gave somewhat similar results to those obtained on rabbits, we suggest that the presence of an inner secretion from the prostate, which influences nitrogen metabolism, is indicated by these experiments.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work, and to them our thanks are due. We wish also to express our sincere gratitude to Prof. C. J. Martin for his continuous support and criticism of this paper.

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THE STANDARDIZATION OF ANTI-DYSENTERY (SHIGA) SERUM.—II.*

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DETAILS of the method used for the standardization of anti-dysentery serum by intravenous injection into mice have already been published (Sudmersen, Runge and O'Brien, 1924), and the standard materials, "toxin" and antitoxin, described. We adopted a fixed dose, 0·025 c.c. of serum C. 950, as a test-dose and titrated the first standard toxin A.A. against this dose, using a large number of mice. The smallest amount of toxin, which, when mixed with the test-dose of serum, would kill approximately 100 per cent. of mice, was taken as the test-dose of toxin A.A. A second toxin, 3 A., has now been similarly titrated against the test-dose of C. 950, and a second serum, S. 9, standardized against the two toxins. The results of these tests are shown in Table I. Toxin 3 A. is almost exactly equal to toxin A.A. when

TABLE I.—*Interrelation of two "Standard" Anti-Dysentery (Shiga) Toxins and two "Standard" Antitoxins.*

Serum.	Dose c.c.	Toxin.	Dose mgms.			Per cent. deaths.			No. of mice used.
C.950	0·025	A.A.	0·2	0·15	0·1	100	91	51	90
C.950	0·025	3.A.	0·2	0·15	0·1	100	87	56	160
S.9	0·002	A.A.	0·2	—	—	95	—	—	40
S.9	0·002	3.A.	0·2	0·15	0·1	100	97	60	60

TABLE II.—*Tests of Various Anti-Dysentery (Shiga) Sera. Test Dose of Toxin 0·2 mgm.*

No. of serum.	Serum c.c.			Results. Lives/No. injected.			Value.
T	0·1	0·05	0·025	4/4	4/7	1/7	0·1
Frankfurt	0·05	0·025	0·01	8/9	3/9	0/6	0·05
L	0·02	0·01	0·005	6/6	2/8	0/5	0·02
W	0·01	0·005	0·0025	6/6	2/6	0/8	0·01
B	0·005	0·003	0·002	4/4	1/6	0/2	0·005
Varsovie	0·006	0·003	0·0015	5/6	7/9	1/12	0·003
A	0·002	0·001	0·0005	4/4	3/8	0/2	0·002

In the "results," the figure after the stroke gives the total number of mice injected, the figure before the stroke the number surviving.

* The work reported in this paper will form a report to the League of Nations Standardization Committee.

TABLE III.—*Antitoxic Value in Units per c.c. of* 18 *Commercial and Standard Anti-Dysentery (Shiga) Sera.*

Serum.	Units per c.c.	Serum.	Units per c.c.
A	500	G4	40
V and G3	300	H, D and E	30
B, G1 and G5	200	Fr	20
W, G2 and K	100	T	10
L, M and S	50		

tested against either of the two sera. Tables II and III give the results of tests on anti-dysentery sera from various laboratories. In Table II are given the details of a few of the tests in order to indicate the degree of accuracy of the method. Table III gives the general summary. The general scheme for the test was to estimate three particular points, small numbers of mice being used. At the first point 80 to 100 per cent. of the mice are protected against the test-dose 0·2 mgm. of toxin; at the second point between 20 and 50 per cent. are protected, and at the third point less than 20 per cent.

These points represent roughly the L 0* and the L + values of the serum with an intermediate point. Two, three or more mice were injected with the same mixture of toxin and serum, and the test was repeated on several occasions either at exactly the same points or at points very near to the previous one. Thus, where the total number injected is given as four, two mice were used on each of two occasions. Where only two mice are shown at the last point, other tests at lower levels had given no protection. The range from L + to L 0 varies a certain amount in the different samples, the L 0 dose being generally about three to four times the L + dose.

Individual mice vary a good deal in their susceptibility to Shiga toxin, and in addition to this they are delicate animals, and easily succumb to changes of temperature or feeding during a period of observation extending over seven days. In order to test within narrow limits a large number of animals would be necessary. In such an instance as No. 6 on the table there is some doubt as to the value to be assigned. The highest point, 0·006, gives one death in six mice, and the next point, 0·003, two deaths in nine. However, when the details of the tests, put up on three separate occasions, were taken into account, it appeared probable that two of the mice had died from other causes and the second point was taken as the true value. In all other cases there was no difficulty in distinguishing, with the small number of mice used, the points at which the serum was tested.

In the last column of Table II are shown "values." We have for some time past used these values as "units," and propose to continue doing so until the international standard unit is fixed by the League of Nations Committee. Our provisional unit is the amount of serum which will neutralize the test-dose 0·2 mgm. of our standard toxin A.A. (this is approximately ten lethal doses). In Table III we have collected the results of titrations of sixteen sera issued by various laboratories, as well as Frankfort standard serum and

* It must be realized that the use of the symbols L + and L 0 here is not in direct accordance with Ehrlich's use of the terms. Despite the inaccuracy, we would retain previsionally these convenient terms instead of the cumbrous phrases "approximately 80 per cent. protection dose" and "approximately 20 per cent. protection dose."

Varsovie standard kindly sent us by Prof. Kolle and Prof. Hirszfeld respectively. "A" was a concentrated serum, the others unconcentrated. It will be seen that the range of values is undesirably wide. At present it is possible for a clinician to purchase and use two samples of commercial anti-dysentery serum which bear no indication on the label, and therefore would be presumed to be of equal "value," and yet when using the weaker serum he would be giving $\frac{1}{20}$th or $\frac{1}{50}$th of the antitoxin contained in the stronger serum.

It appeared to be desirable to find out how accurately one could titrate the antitoxic value of a standard serum if a moderate number of mice were used. From a review of our general results we had formed the opinion that we might be able to distinguish sera differing by 20 per cent. in value.

Two dilutions of a serum of known strength were made by mixing with normal horse-serum so that they contained 80 per cent. and 64 per cent. of the original serum, and the undiluted serum and the two dilutions were numbered and handed over for test. The strength of the undiluted serum was known, but the identity of the three samples was throughout concealed from the tester. Each serum was tested at three points at 20 per cent. intervals from one another, and the test was repeated three times, the samples being re-lettered between each test. Table IV shows the results obtained. A figure in the table represents the day of death of a mouse, L indicates that the mouse lived.

TABLE IV.—*Tests on three Dilutions of Serum. Test-dose* 0·2 *mgm.* 3 *A.*

	Serum c.c.			Identification by tester.
	0·008	0·0064	0·005	
1st test.				
Sample labelled A .	LLLLL	LLLLL	3444L .	80 per cent.
,, ,, B .	45LLL	33444	34446 .	64 ,,
,, ,, C .	6LLLL	LLLLL	457LL .	100 ,,
2nd test.				
Sample labelled A .	6LLLL	3445L	1133L .	64 per cent.
,, ,, B .	LLLLL	45LLL	244LL .	80 ,,
,, ,, C .	LLLLL	LLLLL	3LLLL .	100 ,,
3rd test.				
Sample labelled K .	36LLL	5LLLL	3345L .	80 per cent.
,, ,, L .	6LLLL	4LLLL	33444 .	64 ,,
,, ,, M .	LLLLL	14LLL	3LLLL .	100 ,,

Summary.

Serum.	Dose c.c. 0·008	0·0064	0·005
	15 mice at each dose. Mice protected.		
100 per cent. .	14 .	13 .	10
80 ,, .	13 .	12 .	4
64 ,, .	11 .	5 .	1

In the first test sample C appeared to be somewhat stronger than A, one death on the sixth day at the highest dose being considered accidental, and B

decidedly the weakest. In the second, C is again the strongest and the other two are in the order B and A. The third test gave the order M, K, L. The order in which the three sera were placed by the tester was correct in each case. In only one test was there any real difficulty in distinguishing between two dilutions. K and L in the third test are separated only by the life of a single mouse, and the decision rested more on the judgment of the tester as to the condition of the mouse than on the figures available. On the other occasions the 20 per cent. decrease in value of the three samples is easily recognizable.

CONCLUSIONS.

1. The antitoxic values of anti-dysentery (Shiga) serum can be determined with a reasonable degree of accuracy by the well-known mouse intravenous method.
2. It is possible with a limited number of mice to distinguish between sera differing in strength by about 20 per cent.
3. There is a wide variation in antitoxic strength between the strongest and weakest antitoxic sera used in clinical work in different parts of the world.

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SUDMERSEN, H. J., RUNGE, B. F., AND O'BRIEN.—(1924) *Brit. J. Exper. Pathol.*, **5**, 100.

ON THE SPECIFICITY OF THE PROTEIN OF HUMAN TEARS.

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IN the course of some experiments in which rabbits were immunized with streptococci which had been dissolved by the lysozyme contained in human tears, it was noticed that the serum from the immunized rabbits gave a precipitate when mixed with the tear-dissolved streptococci, but not with these streptococci dissolved by other means. This suggested to us that the precipitation had nothing to do with the streptococci, but was due to the protein in the tears which had been used to dissolve the streptococci employed for immunization of the rabbit and the tears used to dissolve the cocci employed in the precipitation test. Experiments undertaken to test this supposition form the basis of the present communication.

When the physiological text-books are consulted regarding the nature of the protein of tears little information is to be obtained. Sometimes no mention of the chemical constitution of tears is made, sometimes there is merely a statement that tears contain a small amount of protein, and sometimes it is stated that there is present serum albumen and serum globulin in small amounts.

When human tears are boiled there is a very definite clouding of the fluid, and the same is seen when any of the protein precipitants are added, *e.g.* alcohol, acetone, trichloracetic acid, nitric acid, etc. There is a definite precipitate when human tears are semi-saturated with ammonium sulphate, and when this precipitate is centrifuged out the supernatant fluid can still be shown to have albumen present by boiling or completing the saturation with ammonium sulphate. Human tears, therefore, like human serum, contain coagulable proteins of the nature of albumens and globulins.

METHOD OF PRODUCTION OF AN ANTI-TEAR SERUM.

To enable us to test the specificity of the protein of tears a rabbit was immunized with human tears derived from one of us (V.D.A.).* Six intravenous injections of 0·5 c.c. tears were given at intervals of 5 days. At intervals the rabbit's serum was tested for the presence of precipitins to human tears. The technique adopted was as follows:

Serial dilutions of human tears were made in 0·5 c.c. quantities commencing with a 1 in 4 dilution, and to each of these was added 0·1 c.c. of the rabbit's serum. The controls made were (1) tears 1 in 4 without serum, (2) serum with normal saline in place of tears, and (3) normal rabbit's serum with dilutions of tears.

The tubes were incubated at 45° C. for 4 hours, when a reading was made, and then they were left at room-temperature overnight, when a final reading was made 18 hours after the mixtures were made.

The rabbit before injection and also the other normal rabbits used as controls showed no precipitation with any of the tear dilutions. One week after the first injection of tears the rabbit's serum gave a precipitate with all dilutions of tears up to 1 in 32. With further injections the titre became higher, and 10 days after the last injection of tears it showed a marked opacity when mixed with all dilutions of tears up to 1 in 1024. The precipitation after 4 hours' incubation was evidenced by a definite opacity, but after 18 hours there was well-marked flocculation and sedimentation in all the higher concentrations of tears.

QUESTION AS TO WHETHER THE PRECIPITIN PRODUCED BY THE INJECTION OF HUMAN TEARS REACTS WITH TEARS FROM OTHER MEMBERS OF THE HUMAN SPECIES OR WITH TEARS FROM OTHER ANIMALS.

It is clear, then, that injections of tears are capable of stimulating the production in an animal of substances which have the property of forming a precipitate when mixed with the homologous human tears. Experiments were then carried out to ascertain whether these precipitins were specific to the individual or to the species, or whether the protein of tears was the same in all animals in so far as its precipitinogen was concerned. For this purpose the immune serum was tested against the same human tears which had been used in the immunization of the rabbit, human tears from three other healthy

* The tears were obtained by the introduction into the conjunctival sac of a very small quantity of the juice which can be expelled by squeezing lemon-peel. This induces a copious flow of tears for a short time and leaves no lasting effects.

individuals and tears from various animals.* The results obtained are set forth in Table I, from which it will be seen that the titre of the serum is the same to all the samples of human tears, indicating that there is no difference between individuals of the same species. It will be seen, however, that there is a very definite species specificity, and that although there was precipitation observed with the human tears up to a dilution of 1 in 640, there was only very doubtful precipitation with some of the animals' tears in the strongest concentration tested (1 in 20).

TABLE I.—*Precipitating Action of Anti-tear Precipitin Serum on Homologous and Heterologous Tears. Incubated for 4 hours at 45° C.*

Source of tears.	Dilution of tears. 1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280
Human V. D. A—	+++	++	++	++	+	+	0
Human I. McL—	+++	++	++	++	+	+	0
Human J. K. C—	+++	++	++	++	+	+	0
Human A. F—	+++	++	++	++	+	0	0
Horse	±	0	0	0	0	0	0
Cow	±	0	0	0	0	0	0
Sheep	+	±	0	0	0	0	0
Turkey	0	0	0	0	0	0	0

± = trace of opacity. 0 = no opacity.

In order further to test the specificity of the anti-serum complement fixation tests were carried out, using as antigens the tears of man, horse, cow, sheep and turkey. In carrying out these tests serial dilutions of the antigens were made while the amount of anti-tear serum was kept constant; the hæmolytic system used was that usually adopted for the Wassermann reaction, namely, sheep's red corpuscles, anti-sheep serum and guinea-pig complement. The tests were done using very small volumes, as has been recommended by Fleming for the Wassermann reaction. The results obtained are shown in Table II, and it will be seen that these results confirm those obtained with the precipitin reaction (Table I) in that there is a very marked species specificity, there being some fixation of complement up to a dilution of human tears of 1 in 640, while with the other animals there was no fixation, or only incomplete fixation, in the strongest concentration of tears—1 in 10.

TABLE II.—*Anti-tear Precipitin Serum. Complement Fixation with Human and other Animal Tears.*

Source of tears.	Dilution of tears. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.
Human (V. D. A—)	+	+	+	+	+	AC	±	0
Horse	Trace	0	0	0	0	0	0	0
Cow	±	Trace	0	0	0	0	0	0
Sheep	0	0	0	0	0	0	0	0
Turkey	±	Trace	0	0	0	0	0	0

+ = complete fixation of complement. ± = partial fixation.
AC = almost complete fixation. 0 = no fixation.

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QUESTION AS TO WHETHER THE PROTEIN OF TEARS IS THE SAME AS THAT FOUND IN THE SERUM OR OTHER FLUIDS OR TISSUES OF THE BODY.

To investigate this, precipitin tests were done with the anti-tear serum and various body-fluids and extracts of solid tissues. The results obtained are set forth in Table III.

TABLE III.—*Anti-tear Precipitin Serum. Precipitating Action on other Human Secretions, Excretions and Tissue Extracts, and on Hen's-egg White. Incubated for 4 hours at 45° C.*

Fluid tested.	Dilution of test fluid.								
	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.
Tears (V. D. A—)	+++	+++	+++	++	++	+	+	±	0
Blood-serum (6 samples)	0	0	0	0	0	0	0	0	0
Hydrocele fluid	0	0	0	0	0	0	0	0	0
Normal urine	0	0	0	0	0	0	0	0	0
Albuminous urine	0	0	0	0	0	0	0	0	0
Spinal fluid:									
2 samples	+	0	0	0	0	0	0	0	0
3 „	0	0	0	0	0	0	0	0	0
Saliva	0	0	0	0	0	0	0	0	0
Extract of cartilage	0	0	0	0	0	0	0	0	0
Extract of dura mater	0	0	0	0	0	0	0	0	0
Hen's-egg white	0	0	0	0	0	0	0	0	0

A consideration of this table will show us that while there was a very well-marked precipitation with human tears there was no precipitate with human blood-serum (5 specimens), hydrocele fluid, normal or highly albuminous urine, or extracts of cartilage or dura mater. With cerebrospinal fluid there was no precipitation with 3 samples, while with 2 others there was a slight precipitate with a two-fold dilution, but none in the lesser concentrations.

The results which we have obtained show that in human tears there is protein which, when tested by immunological methods, seems to be common to all samples of human tears, but is different from that of the tears of other animals. The protein of human tears, also, appears to be quite different immunologically from that in human blood-serum or in the other secretions or tissues of man. These findings are in marked contrast with those of Uhlenhuth and others in their work on the proteins of the crystalline lens, but fall more into line with those of Hektoen in connection with the proteins of semen.

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